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FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

The importance of the neurotrophins NGF and BDNF in bladder dysfunction. An experimental study in the rat.

BÁRBARA FILIPA FRIAS GARRIDO
TESE DE DOUTORAMENTO APRESENTADA
À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM
NEUROCIÊNCIAS

Porto, 2013

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

PORTO
2013

DISSERTAÇÃO APRESENTADA À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO PARA A CANDIDATURA AO GRAU DE DOUTOR NO ÂMBITO DO PROGRAMA DOUTORAL EM NEUROCIÊNCIAS.

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Em obediência ao disposto no Decreto-Lei nº 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planeamento e execução das experiências, observação e análise de resultados e participei ativamente na redação de todas as publicações que fazem parte integrante desta dissertação:

1. Frias B, Lopes T, Pinto R, Cruz F, Cruz CD. (2011) *Neurotrophins in the lower urinary tract: becoming of age.* Current Neuropharmacology. 9(4):553-8.

2. Pinto R, Frias B, Allen S, Dawbarn D, McMahon SB, Cruz F, Cruz CD (2010) *Sequestration of Brain Derived Nerve Factor by intravenous delivery of TrkB-Ig_2 reduces bladder overactivity and noxious input in animals with chronic cystitis.* Neuroscience. 166(3):907-16.

3. Frias B, Allen S, Dawbarn D, Charrua A, Cruz F, Cruz CD (2013) *Brain-derived neurotrophic factor, acting at the spinal cord level, participates in bladder hyperactivity and referred pain during chronic bladder inflammation.* Neuroscience. 234:88-102.

4. Frias B, Santos J, Morgado M, Sousa M, Allen S, Cruz F, Cruz CD (2013)*The role of Brain Derived Neurotrophic Factor (BDNF) in the development of neurogenic detrusor overactivity (NDO).* (manuscrito submetido).

5. Frias B, Charrua A, Avelino A, Michel MC, Cruz F, Cruz CD (2012) *Transient receptor potential vanilloid 1 mediates nerve growth factor-induced bladder hyperactivity and noxious input.* BJU Int. 110(8 Pt B):E422-8.

To Prof. Célia Duarte Cruz

(Supervisor)

To Prof. Francisco José Miranda Rodrigues da Cruz

(Co-Supervisor)

In memory of my beloved mum

To my aunt Virginia, Ticha, Hugo and Pedro

To my Friends

Prologue

"If memories are the bookmarks in your life story, how have your memories shaped you?"

at Art Gallery of Ontario, AGO, Toronto, CA

I was not 10 yet when Science struck my life - and the life of my dearest ones - like a lightning bolt. I remember people mentioning words like cancer or Alzheimer, and though I could not comprehend their meaning, I felt those words came always accompanied by suffering and great loss. Puzzled but not dismayed, I became determined to put all my efforts and forward my interest in Science in search for answers.

At school, I was particularly fond of biology and chemistry. I loved being at the laboratory doing all that make-believe experiments that, most of the times, didn't end up as they were supposed to. It was only when I joined college that I truly understand the meaning of Science. In 2003, I started my Microbiology Degree at *Escola Superior de Biotecnologia*, of the *Universidade Católica Portuguesa*. Here, I was given the chance to gain knowledge of different subjects such as microbiology, biochemistry, genetics, virology, among others. I was impressed by the complexity of each theme. Most importantly, it was a fundamental experience that allowed me to get acquainted with varied experimental techniques and acquire the necessary lab skills and expertise. Following my degree, I was determined to acquire a deeper knowledge of health science, and this led me to *Sciences Faculty of the University of Porto*, where I took a master course in Biochemistry. Throughout my master degree, I developed a particular interest for Neurosciences, hence I ended up elaborating my thesis in the group of Prof. Francisco Cruz, at the *Faculty of Medicine of the University of Porto*. Back then, in 2008, I knew little about neurotrophins and their role in the micturition reflex. However, I was thrilled with the opportunity to embrace and participate in such a good project. After the conclusion of my master degree in Biochemistry, I decided to pursue my research on understanding the effects of neurotrophins in the development of bladder dysfunction and visceral pain as my PhD thesis subject. Looking back in time, it is impossible not to be grateful to the people who supported me and helped me fulfilling this work in every way possible.

First of all, I would like to thank Prof. Celia D. Cruz for accepting me as her student and for supervising this thesis. Without her determinant strength, patience, persistence and assertive advice, this thesis would not have been done. I thank her for the continuous encouragement, guidance and support both at scientific and personal level.

I must express all my gratitude to Prof. Francisco Cruz for having kindly welcomed me in his research team and introduced me to the world of Neurourology. I will always be grateful for his critical thinking, leadership and creative insights, for his fundamental support in a particular difficult time in my life, and for his ability to build a rigorous and at the same time joyful working environment.

A special thank you to Prof. Ana Charrua for shedding light into some scientific questions, for sharing and stimulating new ideas, for her companionship, patience and attention. She has been a role model for her interest in science, her integrity and dedication. I must also thank Prof. Antonio Avelino for his critical thinking and scientific enlightenment and discussions.

I would also like to thank Prof. Deolinda Lima who allowed me to integrate the PhD Programme in Neurosciences and to perform my laboratory work at the Department of Experimental Biology. I thank her for the constructive critiques and the new ideas shared during *science at lunch* discussions.

All my gratitude goes also to Dr. Shelley Allen and everyone at her laboratory at the Molecular Neurobiology Unit, University of Bristol, School of Clinical Sciences, Dorothy Hodgkin Building, in Bristol for kindly giving me the home made recombining proteins, TrkA-Ig_2 and TrkB-Ig_2. This work would have not been possible without them.

I wish to demonstrate my deep admiration and gratitude to Prof. Martin Michel for receiving me in his laboratory at the Department of Pharmacology and Pharmacotherapy, AMC, in Amsterdam. His critiques and informal way of dealing with Science made everything look easy. I must also thank Prof. Peter Ochodnicky for his supervision during my stay and for having introduced me to the world of cell culturing. Many thanks to my friend Hana Cernecka for accompanying me in the discovery of The Netherlands during weekends.

I would also like to express my gratitude and admiration to Prof. Monica Sousa for receiving and allowing me to perform part of my experimental tasks in her laboratory at IBMC- *Instituto de Biologia Molecular e Celular.* I would also like to thank her student, Marlene Morgado, for helping me with the technique and accompanying me during my stay at IBMC.

I would like thank to Prof. Fani Neto, Prof. Carla Morgado, Prof. Jorge Ferreira, Prof. Alexandra Gouveia and Prof. Adriana Rodrigues for their advice and clarification of technical doubts.

I must not forget my awesome lab mates Ana Coelho, Isabel Regadas, Mariana Matos, Maria Ângela Ribeiro, Diana Sousa, Diana Nascimento, Margarida Oliveira, Gisela Borges, Patricia Terra, Sérgio Barros, José Pedro Castro and César Monteiro for their constant good mood and for being good friends. The work in this thesis was also possible thanks to the technical support of D. Elisa Galvão and Fernando Martins. Also, a special thank to Dr. Luisa Guardão, our dedicated and weariless veterinary, and to Liliana e Zita, the fabulous caregivers from the animal house facilities from the *Faculty of Medicine of University of Porto*.

I would like to thank to João Santos, who despite having a lot in hands with his medical classes, was kindly enough to arrange some time and come to the laboratory, helping me in some of the most important experiments. Thank you for your good mood and friendship.

A special thank you to my dearest friends Ana Brandão, Vanessa Ochoa, Paula Parreira e Guerra, Marina Paulo, Joana Senra, Manuela Gonçalves and Catarina Magalhães. Thank you for your words of encouragements and advice. You will always be my sweetheart friends!

To my family, my aunt Virginia and my cousins Ticha, Hugo and Pedro: thank you with all my heart for your support and words of advice.

And above all, I thank my beloved mother for making me who I am today: you will always be my shelter and guardian angel.

Finally, I thank Science for being part of my life.

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Sumário e Conclusões

As neurotrofinas são uma família de fatores de crescimento. Estas proteínas desempenham um papel importante durante o desenvolvimento e na idade adulta na regulação da sobrevivência, crescimento neuronal, plasticidade sináptica e neuroprotecção das populações neuronais dos sistemas nervoso central e periférico. Neste estudo, investigou-se o papel das neurotrofinas Factor de Crescimento Nervoso (NGF, do inglês *Nerve Growth Factor*) e Factor Neurotrófico Derivado do Cérebro (BDNF, do inglês *Brain Derived Neurotrophic Factor*) na dor visceral e hiperactividade vesical associadas à cistite, bem como o papel do BDNF na hiperactividade neurogénica do detrusor (NDO, do inglês *Neurogenic Detrusor Overactivity*).

Os dois primeiros estudos avaliaram o papel do BDNF como mediador dos estímulos sensitivos nóxicos produzido pela bexiga e da hiperactividade vesical, usando um modelo animal de cistite induzida com ciclofosfamida. A administração intratecal de BDNF a animais intactos causou dor referida no abdómen e na pata, bem como um aumento da frequência das contrações reflexas da bexiga imediatamente após injeção. Os efeitos agudos da administração intratecal de BDNF na função vesical e sensibilidade cutânea foram de curta duração. Curiosamente, o tratamento crónico com BDNF provocou hipersensibilidade cutânea sem qualquer efeito na função miccional, evidenciando assim a importância da componente inflamatória para o desenvolvimento disfunção vesical associada à inflamação da bexiga. Nos ratos com cistite induzida com ciclofosfamida, os níveis de BDNF estavam aumentados quer na bexiga quer na medula espinhal. Estes animais apresentavam hipersensibilidade cutânea e hiperactividade vesical. A administração intratecal e intravenosa de TrkB-Ig_2, um sequestrante de BDNF, reduziu significativamente a dor referida e a disfunção miccional. Estas alterações foram acompanhadas por um decréscimo da expressão espinhal de c-Fos e da forma fosforilada da proteína ERK. Apesar disso, não houve redução da inflamação da bexiga, o que sugere que o BDNF produzido na periferia não participa na inflamação (publicação II e III). ***(Pinto and Frias et al. 2010. Neuroscience, 166:907-916; Frias et al. 2013. Neuroscience, 234:88-102).***

O terceiro estudo focou-se na contribuição do BDNF para o estabelecimento e manutenção NDO. Os dados obtidos mostraram que o aparecimento da hiperactividade vesical foi acompanhado por um aumento dos níveis de BDNF ao longo do tempo, maioritariamente na lâmina I e II do corno dorsal da medula espinhal. A sequestração de BDNF, iniciada imediatamente após lesão da medula espinhal, induziu o estabelecimento precoce de NDO juntamente com o aumento da capacidade de crescimento dos neurónios ganglionares da raiz dorsal. Estes dados indicam que o BDNF terá um papel protetor na função vesical em casos de lesão da medula espinhal. Para confirmar esta hipótese, foi administrado BDNF por via intratecal a animais com lesão medular, tendo o tratamento se iniciado logo após lesão.

Registou-se uma melhoria da função miccional após quatro semanas de administração de BDNF. Em ratos espinalizados com NDO crónica, a sequestração de BDNF reduziu a disfunção vesical, evidenciado pela redução da frequência e amplitude da atividade reflexa da bexiga. Tal sugere que o BDNF pode ter diferentes intervenções durante a progressão da doença. Estes resultados implicam marcadamente esta neurotrofina como regulador do aparecimento e manutenção da NDO. Assim, o BDNF poderá ser um alvo terapêutico atrativo para ser manipulado nos diferentes estadios da NDO (publicação IV). ***(Frias et al., submitted).***

O último estudo avaliou o papel do TRPV1 (do inglês, *Transient receptor potencial vanilloid 1*) nos efeitos excitatórios provocados pela administração crónica de NGF na atividade reflexa da bexiga e de estímulos sensitivos. Os resultados obtidos mostraram que a administração crónica de NGF induziu hiperactividade da bexiga e hipersensibilidade a estímulos térmicos em animais WT (do inglês, *Wild-Type*). Os ratinhos TRPV1 KO (do inglês, *Knock-out*) não apresentaram qualquer alteração, quer a nível da função miccional quer na sensibilidade cutânea. Estes resultados indicam que o recetor TRPV1 é essencial para os efeitos produzidos pela administração de NGF na função vesical e sensibilidade cutânea. Em suma, estes resultados indicam que a interação entre o NGF e o TRPV1, que se sabe ser essencial para o desenvolvimento da dor somática, também é importante para a regulação da função vesical e dor visceral. Assim, o recetor TRPV1 é um elemento crucial no aparecimento da disfunção vesical em patologias associadas a uma sobre-expressão de NGF (publicação V). ***(Frias et al. 2012. BJU Int, 110:E422-E428)***.

Summary and Conclusions

Neurotrophins (NTs) are a family of growth factors characterized for their important roles on survival, neurite outgrowth, synaptic plasticity and neuroprotection of neuronal populations from central and peripheral nervous systems during development and adulthood. In this study, we focused our research on Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF) actions and we investigated their contribution in the regulation of bladder reflex activity as well as their role in visceral pain. We used animal models of chronic bladder inflammation and spinal cord injury.

The first two studies addressed the effects of BDNF as a mediator of bladder-generated noxious input and bladder overactivity using an animal model of CYP-induced cystitis. Intrathecal administration of BDNF to intact animals caused pain and increased the frequency of bladder reflex contractions immediately after injection. The acute effects of intrathecal administration of BDNF for bladder function and pain were short-lived. Interestingly, chronic BDNF treatment caused cutaneous hypersensitivity without affect bladder function, stressing the importance of the inflammatory component in inflammation-dependent bladder dysfunction. In rats with CYP-induced cystitis, BDNF was upregulated both in the bladder as in the spinal cord. These animals presented cutaneous hypersensitivity and bladder hyperactivity. Intrathecal and intravenous delivery of TrkB-Ig_2 significantly reduced the behavioral signs of pain and bladder dysfunction. This was accompanied by a decrease in the spinal expression of c-Fos and phosphoERK. Because no reduction of inflammatory signs was observed, it is unlikely that peripheral BDNF participates in inflammation (publications II and III). ***(Pinto and Frias et al. 2010. Neuroscience, 166:907-916; Frias et al. 2013. Neuroscience, 234:88-102).***

The third study focused on the putative contribution of BDNF for the establishment and maintenance of neurogenic detrusor overactivity (NDO). The data obtained showed that the emergence of bladder hyperactivity was accompanied by a time-dependent increase in BDNF expression mostly in the laminae I and II of the dorsal horn of the spinal cord. BDNF scavenging, initiated immediately after spinal injury, induced the early establishment of NDO which correlated with increased growth capacity of DRG neurons. This indicates that BDNF may have a protective role on bladder function in SCT. To confirm this, spinal cord injured rats were treated with intrathecal BDNF. Treatment was initiated immediately after cord injury. Improvement of bladder function was only observed after four weeks of BDNF administration. In rats with established NDO, BDNF sequestration ameliorated bladder dysfunction by reducing the frequency, amplitude and peak pressure of reflex contractions, suggesting that BDNF may have a differential role during disease progression. These findings identify and strongly imply that this NT participates in the emergence and maintenance of NDO. Thus,

BDNF is an attractive therapeutic target to be differentially manipulated at different stages of NDO establishment (publication IV). ***(Frias et al., submitted).***

The last study evaluated the role of TRPV1 in the excitatory effects of chronic administration of NGF on bladder generated sensory input and reflex activity. The data obtained showed that chronic administration of NGF induced bladder hyperactivity and thermal hypersensitivity in WT animals. This was not observed in TRPV1-KO mice, indicating that this receptor is essential for NGF-dependent bladder dysfunction and pain. Overall, these results indicate that the interaction between the NGF and TRPV1 systems, essential for somatic pain, remains operative in the regulation of bladder function and visceral pain. Thus, TRPV1 receptor is an important bottleneck in bladder hyperactivity in pathologies associated with NGF overexpression (publication V). ***(Frias et al. 2012. BJU Int, 110:E422-E428)***.

List of Abbreviations

ASIC2 – Acid Sensing Ionic Channel 2

BDNF – Brain-Derived Neurotrophic Factor

BPS/IC – Bladder Pain Syndrome/Interstitial Cystitis

CFA – Complete Freund's Adjuvant

CGRP – Calcitonin Gene-Related Peptide

CYP – Cyclophosphamide

DCM – Dorsal Comissure

DH – Dorsal Horn

DRG – Dorsal Root Ganglia

ERK – Extracellular signal-Regulated Kinases 1 and 2

ILGs – Intermediolateral Grey Matter areas

MAPK – Mitogen-Activated Protein Kinase

NDO – Neurogenic Detrusor Overactivity

NGF – Nerve Growth Factor

NMDA – N-methyl-D-aspartate

NT-3 – Neurotrophin 3

NT-4/5 – Neurotrophin 4/5

NTs - Neurotrophins

OAB – Overactive Bladder Syndrome

PKC – Protein Kinase C

Trk Receptor – Tyrosine Kinase Receptor

TrkB-Ig_2 – TrkB Immunoglobulin-like protein

TRPV1 – Transient Receptor Potential Vanilloid 1

TRPV1 KO – TRPV1 knock-out

TTX-R sodium currents – Tetrodotoxin-resistant sodium currents

WT – Wild Type

Introduction

(Based on Frias et al (2011). Neurotrophins in the lower urinary tract: becoming of age. Curr Neuropharmacol. 9(4):553-8)

1. Neurotrophins and their receptors

bŜdzN2ΩN2LJKƛyã ób¢ăú IּNŜ Iּ ŦIּYˉƛfè 2Ŧ ƎN2ø΄K ŦIּŎΩ2Nă LJN2ŘdzŎŜŘ ōè ŜLJƛΩKŜfΩIּ IּyŘ 2ΩKŜN ΩƛăădzŜăΦ LyˉYIּYYIּfãΣ ΩKŜNŜ IּNŜ Ŧ2dzN ˥y2øyˉ b¢ăY bŜNØŜ DN2øΩK CIּŎΩ2N óbDCΩΣ .NIּƛyˉ5ŜNƛØŜŘ bŜdzN2ΩN2LJKƛŎ CIּŎΩ2N ó.5bCΩΣ bŜdzN2ΩN2LJKƛyˉo ób¢-oú IּyŘ bŜdzN2ΩN2LJKƛyˉ-пкр ób¢-пкрúΦ ¢KŜ Yˉ2ăΩ øŜft-˥y2øyˉ b¢ăΣ bDC IּyŘ .5bCΣ LJfIּè Iּ ŎNƛΩƛŎIּf N2fŜ ŘdzNƛyƎ ŜYˉōNè2f2ƎƛŎIּf ŘŜØŜf2LJYˉŜyΩ IּyŘΣ ŘdzNƛyƎ IּŘdzfΩK22ŘΣ ƛyˉ ΩKŜ NŜƎdzfIּΩƛ2yˉ 2Ŧ ySdzN2yIּf ădzNØƛØIּfΣ ySdzNƛΩŜ 2dzΩƎN2øΩKΣ IּyŘ ăèyIּLJΩƛŎ LJfIּăΩƛŎƛΩè 2Ŧ ŘƛŦŦŜNŜyΩ ySdzN2yIּf LJ2LJdzfIּΩƛ2yã f2ŎIּΩŜŘ ō2ΩK ƛyˉΩKŜ ŎŜyΩNIּf IּyŘ LJŜNƛLJKŜNIּf ySNØ2dză ăèăΩŜYˉă óIdzIּyƎ IּyŘ wŜƛŎKIּNŘΩΣ нллмΣ aŜNƛƎKƛ ŜΩ IּfΦΣ ннллпΣ tŜÎŜΩ IּyŘ aŎaIּK2yΣ нллсúΦ b¢ă YIּè Iּfã2 LJIּNΩΩƛŎƛLJIּΩŜ ƛyˉ ŘƛØŜNăŜ ŘƛăŜIּăŜă ăΩIּΩŜă ƛyŎfdzŘƛyƎ !fÎKŜƛYˉŜNΩă ŘƛăŜIּăŜΣ IdzyΩƛyƎΩ2yΩă ŘƛăŜIּăŜΣ ŘŜLJNŜăăƛ2yΣ ŎKN2yIŎ LJIּƛyˉ IּyŘ IּăΩKYIּ ó5Iּøōl·NyˉIּyŘ !ffŜyΣ нллоΣ !ffŜyˉIּyŘ 5Iּøōl·NyΣ нллсúΦ

b¢ă IּNŜ ŘƛYˉŜNƛŎ LJN2ΩŜƛyã øƛΩK Iּ Yˉ2fŜŎdzfIּN øŜƛƎKΩ 2Ŧ ŎƛNŎIּ мн˥5Iּ ó[ŜøƛyˉIּyŘ .IּNŘŜΣ мффсΣ a2øfIּ ŜΩ IּfΦΣ ннллмΣ tŜÎŜΩ IּyŘ aŎaIּK2yΣ нллсΣ hŎK2ŘyƛŎ˥è ŜΩ IּfΦΣ нлмнúΦ !ff b¢ă IּNŜ ăèyΩKŜăƛÎŜŘ Iּă LJNŜLJN2LJN2ΩŜƛyã 2Ŧ нрл IּYˉƛy2IּŎƛŘă ƛyˉ ΩKŜ ŜyŘ2LJfIּăYIּΩƛŎ NŜΩƛŎdzfdzYΦ ¢KŜ LJNŜ-Ř2YIּƛyˉ ăă ŎfŜIּØŜŘ ƛYYˉŜŘƛIּΩŜfè IּŦΩŜN ăèyΩKŜăƛă IּyŘ ΩKŜ NŜădzfΩƛyƎ LJN2LJN2ΩŜƛyˉ YIּè dzyŘŜNƎ2 LJ2ăΩΩNIּyãfIּΩƛ2yIּf Yˉ2ŘƛŦƛŎIּΩƛ2yã ƛyˉ ΩKŜ D2fƎƛ IּLJLJIּNIּΩdză IּyŘ ΩNIּyã-D2fƎƛ ySΩø2N˥ ōŜŦ2NŜ ōŜƛyƎ ăΩ2NŜŘ ƛyˉ ăŜŎNŜΩ2Nè ØŜăƛŎfŜă¢ ¢KŜăŜ ØŜăƛŎfŜă IּNŜ ΩKŜyˉ ă2NΩŜŘ ŜΩƛŎKŜN ƛyΩ2 ΩKŜ NŜƎdzfIּΩŜŘ /Iּ[Hb]-ŘŜLJŜyŘŜyΩ LJIּΩKøIּè 2N ƛyΩ2 ΩKŜ Ŏ2yãΩΩdzΩƛØŜ LJIּΩKøIּè¢ /fŜIּØIּƎŜ 2Ŧ ΩKŜ LJN2-Ř2YIּƛyˉ2ŎŎdzNă ƛyˉ ΩKŜ ŜÈΩNIּŎŜffdzfIּN ălJIּŎŜ ōè YIּΩNƛÈ LJN2ΩŜIּăŜă IּyŘ LJN2ŘdzŎŜă ΩKŜ YIּΩdzNŜ Ŧ2NYˉ 2Ŧ b¢ă ó[ŜăăYIּyYˉ IּyŘ .NƛƎIּŘă˥λΣ нллфú¢

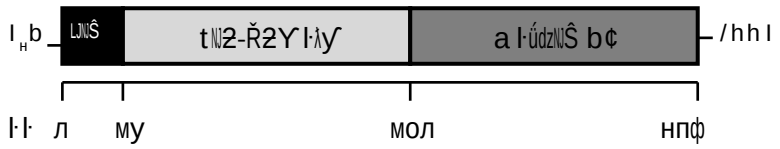

Figure 1. The different domains of neurotrophins are shown along the length of its aminoacids sequence (adapted from (Lessmann and Brigadski, 2009).

¦LJ2yˉ NŜfŜIּăΣ b¢ă YIּè ōƛyŘ Ω2 Ωø2 ŘƛŦŦŜNŜyΩ NŜŎŜLJΩ2N ΩèLJŜăY ΩKŜ KƛƎK IּŦŦƛyΩΩè ΩNΩ2LJ2Yè2ăƛyˉNΩfIּΩŜŘ ˥ƛyIּăŜ ó¢N˥ó NŜŎŜLJΩ2Nă IּyŘ ΩKŜ f2ø IּŦŦƛyΩΩè ySdzN2ΩN2LJKƛyˉNŜŎŜLJΩ2NΣ LJтр$^{b¢w}$Φ ² KŜNŜIּă Iּfb¢ă YIּè ŜIjdzIּffè Ŏ2yyŜŎΩ Ω2 LJтр$^{b¢w}$Σ ¢N˥ NŜŎŜLJΩ2N øƛff 2yfè ōƛyŘ Ω2 2yŜ ăLJŜŎƛŦƛŎ b¢Φ ¢N˥! ăă ΩKŜ KƛƎK-IּŦŦƛyΩΩè NŜŎŜLJΩ2N Ŧ2N bDCΦ .5bC IּyŘ b¢пΣ IּfōŝƛΩ øƛΩK fŜăă IּŦŦƛyΩΩèΣ ōƛyŘ Ω2 ¢N˥. IּyŘ b¢o ōƛyŘă Ω2 ¢N˥/ ótŜÎŜΩ IּyŘ aŎaIּK2yΣ нллсΣ hŎK2ŘyƛŎ˥è ŜΩ IּfΦΣ нлмнúΦ ¢N˥ IּyŘ LJтр$^{b¢w}$ NŜŎŜLJΩ2Nă IּNŜ 2ŦΩŜyˉLJNŜăŜyΩ 2yˉΩKŜ ăIּYŜ ŎŜff IּyŘ Yˉ2ŘdzfIּΩŜ ΩKŜ NŜăLJ2yãŜă Ω2 b¢ă óIdzIּyƎ IּyŘ

wŜƛǑKI·₦ŘűΣ нллоú¢ Lŧ úKŜ ₦I·úƛ2 LJтр$^{b¢w}$к¢₦ ƛă KƛƎK 2₦ ƛŧ LJтр$^{b¢w}$ ƛă ŜÈLJ₦ŜăăŜŘ ƛy¯úKŜ I·ōăŜyÖŜ 2ŧ ¢₦ΣΣ ōƛyŘƛyƎ 2ŧ b¢ă Y·l·è LJ₦2Y¯2úŜ l·LJ2LJú2ăƛă¢ Ly¯Ô2yú₦I·ăűΣ ƛŧ úKŜ ₦I·úƛ2 LJтр$^{b¢w}$к¢₦ ƛă f2ǿΣ ōƛyŘƛyƎ 2ŧ úƛăădzŜ ŘŜ₦ΛØŜŘ b¢ă ű2 úKŜƛ₦ ăLJŜǑƛŧΛǑ ¢₦ ₦ŜǑŜLJú2₦ øλff LJ₦2Y¯2ûŜ ǑŜff ădz₦ØΛØI·f ó l dzl·yƎ l·yŘ wŜƛǑKI·₦ŘűΣ нллоΣ l Ŝŧúƛ Ŝű l·f¢Σ нллсú¢

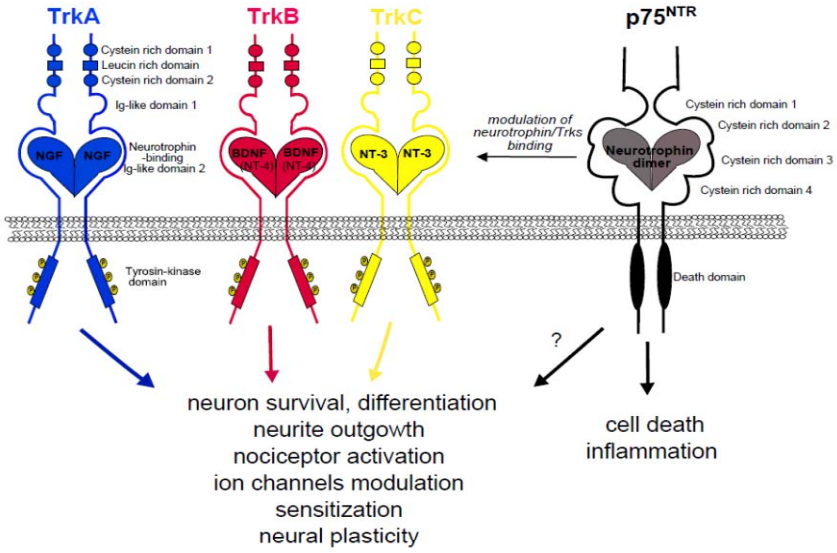

Figure 2. Schematic structure of mammalian neurotrophin receptors and consequences of receptor-ligand binding.

.ƛyŘƛyƎ 2ŧ b¢ă ű2 úKŜƛ₦ KƛƎK-I·ŧŧƛyƛúě ¢₦ΣΣ ₦ŜǑŜLJú2₦ ƛă ₦ŜƎdzfl·úŜŘ ōè I· ₦ŜƎƛ2y¯2ŧ Ǒ2yãŜ₦ØΛŜŘ I·yŘ ØI·₦ΛI·ōfŜ Ř2YI·ƛyã úKI·ű I·ff2ǿ úKŜ ăLJŜǑƛŧΛǑ ₦ŜǑ2Ǝyƛúƛ2y¯ 2ŧ ŜI·ǑK ySdz₦2ú₦2LJKƛy¯ ó l dzl·yƎ l·yŘ wŜƛǑKI·₦ŘűΣ нллоΣ !₦ŜØI·f2 I·yŘ ² dzΣ нллсú¢ !ă ŧ2₦ LJтр$^{b¢w}$Σ b¢ ōƛyŘƛyƎ ƛă Y¯ŜŘƛI·úŜŘ ōè ű ǿ2 ÔèăúŜƛy¯ ₦ŜLJŜI·ű Ř2YI·ƛyã ó/wн I·yŘ /woúΣ f2ÔI·úŜŘ ƛy¯úKŜ ŜÈú₦I·ÖŜffdzfl·₦ Ř2YI·ƛy¯2ŧ úKŜ ₦ŜǑŜLJú2₦¢ !ÖűΛØI·ûΛ2y¯ 2ŧ ¢₦ΣΣ tŜI·Řă ű2 LJK2ăLJK2₦èfI·ûΛ2y¯2ŧ ŘƛŧŧŜ₦Syú ûè₦2ăƛyŜ ₦ŜăƛŘdzŜă 2y¯úKŜ Ôèű2LJfI·ăY¯I·ûΛǑ Ř2YI·ƛyã 2ŧ úKŜ ₦ŜǑŜLJú2₦ă I·yŘ Ř2øyãú₦ŜI·Y¯ I·ÖűΛØI·ûΛ2y¯2ŧ ƛyú₦I·ǑŜffdzfl·₦ ăƛƎyI·fƛyƎ LJI·úKǿI·èă ƛyǑfdzŘƛyƎ a ! tYΣ tLoYκ!ΣΣű I·yŘ tY/ ó l dzl·yƎ I·yŘ wŜƛǑKI·₦ŘűΣ нллоΣ !₦ŜØI·f2 I·yŘ ² dzΣ нллсΣ tŜÎŜú I·yŘ aǑa I·K2yΣ нллсú¢

DƛØŜy¯úKŜ Y¯2ŘŜăú ₦2fŜ 2ŧ b¢-o I·yŘ b¢-пкр ƛy¯LJI·ƛy¯ LJ₦2ǑŜăăƛyƎ I·yŘ ₦ŜƎdzfl·ûΛ2y¯2ŧ ōfI·ŘŘŜ₦ ŧdzyÖûΛ2yΣ úKŜ LJ₦ŜăŜyú ₦ŜØΛŜǿ øλff 2yfè ŧ2Ödză 2y¯ bDC I·yŘ .5bC¢ CdzNúKŜ₦ ƛyŧ2₦Y¯I·ûΛ2y¯ I·ō2dzű úKŜăŜ b¢ă Y¯I·è ōŜ ŧ2dzyŘ ŜfăŜǿKŜ₦Ŝ ó!yΣI·yŘΣ нллпΣ /₦dzÎΣ нлмоú¢

2. Nerve Growth Factor (NGF)

bŜ₦ØŜ D₦2ǿúK CI·ÖűΛ2₦ óbDCú ƛă úKŜ ŧ2dzyŘƛyƎ Y¯ŜY¯ōŜ₦ 2ŧ úKŜ b¢ă ŧI·Y¯ƛfè¢ Lű ǿI·ă ŘƛăÖ2ØŜ₦ŜŘ ƛy¯úKŜ мфрлΩă I·ă I· Ǝ₦2ǿúK ŧI·Ôű2₦ ₦ŜIjdzƛ₦ŜŘ ŧ2₦ I·È2yΣI·f Ǝ₦2ǿúK 2ŧ Y¯2ú2₦ I·yŘ ăŜyã2₦è ySdz₦2yã ƛy¯úKŜ ÖKƛÖΣΣ ó l I·Y¯ōdz₦ƎŜ₦ I·yŘ [ŜØƛ-a2yúI·fÖƛyΛΣ мфпфΣ [ŜØƛ-a2yúI·fÖƛyΛ I·yŘ l I·Y¯ōdz₦ƎŜ₦Σ мфрмΣ [ŜØƛ-

Montalcini and Angeletti, 1966, Levi-Montalcini, 1975, 1987, Ernsberger, 2009). NGF is presently recognized as an essential trophic protein regulating the development and survival of small diameter primary dorsal root ganglia (DRG) neurons, as well as sprouting of postganglionic sympathetic neurons, as they abundantly express TrkA (Levi-Montalcini, 1987, Pezet and McMahon, 2006). In peripheral tissues, NGF may be synthesized by non-neuronal cells, including cells from the salivary glands (Watson et al., 1985, Nam et al., 2007), smooth muscle cells (Steers et al., 1991), immune cells (Aloe et al., 1992, Hefti et al., 2006) and epithelial cells (Pincelli and Marconi, 2000, Harrison et al., 2004, Birder et al., 2007, Stanzel et al., 2008). NGF is uptaken by the peripheral neuronal terminals and retrogradely transported to the cell bodies of dorsal root ganglia (DRG) neurons, where it activates pro-survival intracellular programs. Although normal levels are low in adult tissue, they are sufficient to exert protective effects on peripheral innervation.

2.1. NGF as a pain mediator

An important function of NGF is the regulation of pain-signaling systems as NGF is essential for the survival and plastic changes of peripheral nociceptive neurons as well as the generation and maintenance of chronic pain states (McMahon, 1996, Hunt and Mantyh, 2001, Julius and Basbaum, 2001). NGF is upregulated in a variety of chronic painful conditions, including arthritis (Aloe et al., 1992, Halliday et al., 1998, Iannone et al., 2002), cystitis (Lowe et al., 1997, Oddiah et al., 1998, Jaggar et al., 1999, Liu et al., 2009), prostatitis (Miller et al., 2002) and chronic headaches (Sarchielli et al., 2001). Healthy volunteers reported cutaneous hypersensitivity and generalized pain starting within minutes after subcutaneous or muscular administration of NGF and persisting for several hours (Petty et al., 1994, Dyck et al., 1997, Svensson et al., 2003, Andersen et al., 2008, Hoheisel et al., 2013). Likewise, subcutaneous injection of NGF in the hindpaw of rodents lead to increased responsiveness to noxious heat and progressive increase in the activity of nociceptive neurons (Lewin et al., 1994, Andreev et al., 1995, Thompson et al., 1995). Also in rodents, the concentration of NGF in the skin increases in response to inflammation produced by injection of irritants, such as complete Freud's Adjuvant (CFA) (Pezet and McMahon, 2006), or following exposure to ultraviolet-B irradiation (Woolf et al., 1994, Bishop et al., 2007). Importantly, NGF has also been linked to visceral pain (Guerios et al., 2006). Finally, it has been recently demonstrated that NGF is an important mediator in experimental models of cancer pain (Sevcik et al., 2005, Bloom et al., 2011, Jimenez-Andrade et al., 2011).

Following the demonstration of high levels of NGF both in experimental models and in human painful conditions, several studies addressed the effects of the downregulation of this

NT. The administration of anti-NGF antibodies, synthetic NGF scavengers and antagonists of Trk receptors successfully reduced pain levels following intraplantar injection of CFA (Lewin et al., 1994, Woolf et al., 1994, Ma and Woolf, 1997), carrageenan-induced inflammation of the hindpaw (McMahon et al., 1995, Chudler et al., 1997, Sammons et al., 2000) and bladder inflammation (Hu et al., 2005, Guerios et al., 2008). Following the demonstration that NGF is a major mediator of pain and reports of the beneficial effects of NGF blockade, a recombinant humanized monoclonal antibody against NGF was developed and tested in preclinical studies to treat pain in osteoarthritic patients (Cattaneo, 2010, Lane et al., 2010, Nagashima et al., 2011, Schnitzer et al., 2011, Brown et al., 2012, 2013). Despite positive effects, patients also developed adverse side problems, including asseptic avascular bone necrosis that eventually lead to joint replacement (Brown et al., 2013), precluding its investigation in other painful conditions.

The reason why NGF is such an important pain mediator is related to its downstream effects (McMahon, 1996, Pezet and McMahon, 2006). Upon release from peripheral tissues, NGF is retrogradely transported to the cell soma of sensory neurons, where it activates signaling pathways such as the Extracellular regulated kinase 1 and 2 (ERK) and Akt signalling pathways (Pezet and McMahon, 2006, Cruz and Cruz, 2007, Ochodnicky et al., 2012). Activation of these signaling pathways reduces the activation threshold of sensory afferents either by a quick and direct modulation of membrane receptor or by inducing long-lasting changes in gene expression. Examples of genes regulated by NGF include the ones encoding for other neurotrophins, such as BDNF, and ionic channels, such as the Transient Receptor Potential Vanilloid 1 (TRPV1) (Pezet and McMahon, 2006). Interestingly, acute NGF-induced sensitization may be accounted by a direct interaction between NGF and TRPV1. TrkA activation, induced by NGF binding, leads to the release of TRPV1 from tonic inhibition (Chuang et al., 2001) and quickly increases TRPV1 expression by inducing the insertion in the membrane of new TRPV1 subunits (Zhang et al., 2005, Zhang et al., 2008). However, the interaction between TRPV1 and the NGF system has only been analysed in *in vitro* assays and in the context of somatic pain.

2.2. NGF in the Lower Urinary tract (LUT)

In the LUT, NGF is by far the most well studied neurotrophin. Early studies showed an increase of NGF levels and hypertrophied bladders in rats with urethral obstruction (Steers et al., 1991) and proved NGF as an essential mediator in regulating survival and neurite outgrowth of cultured major pelvic ganglion neurons (Tuttle and Steers, 1992, Tuttle et al., 1994a, Tuttle et al., 1994b). Other studies aimed to detect the origin of NGF in the bladder and

identified bladder smooth cells (Persson et al., 1997, Tanner et al., 2000) and the urothelium (Lowe et al., 1997, Birder et al., 2007, Birder et al., 2010) as important sources of NGF. Interestingly, urothelial cells also respond to this NT as they also express TrkA and $p75^{NTR}$ (Murray et al., 2004). In addition, NGF can also been found in the cell bodies of sensory afferents innervating the urinary bladder (Sasaki et al., 2002) and major pelvic ganglia (Murray et al., 2004).

2.2.1 NGF and bladder dysfunction

NGF is upregulated in the bladder in various human LUT pathologies including Bladder Pain Syndrome/Interstitial Cystitis (BPS/IC), Overactive Bladder Syndrome (OAB), Bladder Outlet Obstruction (BOO) and Neurogenic Detrusor Overactivity (NDO) (Ochodnicky et al., 2011, Antunes-Lopes et al., 2013). This NT is found in big amounts in the urines of these patients, subsiding after pharmacological and non-pharmacological treatment (Pinto et al., 2010b, Antunes-Lopes et al., 2011, Antunes-Lopes et al., 2013). Collectively, these studies raised the ongoing debate about a putative use of urinary NGF as a biomarker of bladder dysfunction (Liu and Kuo, 2008, Ochodnicky et al., 2011, Seth et al., 2013).

In experimental animals of bladder hyperactivity, NGF concentration in the bladder is increased in animals with cystitis (Bjorling et al., 2001, Guerios et al., 2008) and following spinal cord injury (SCI) (Vizzard, 2000). Exogenous administration of NGF to the bladder leads to hyperactivity, with increased frequency and amplitude of bladder contractions, irrespective of the route of administration (Dmitrieva and McMahon, 1996, Dmitrieva et al., 1997, Lamb et al., 2004, Zvara and Vizzard, 2007). More recently, it was reported that transgenic mice with NGF overexpression restricted to the urothelium also present bladder hyperactivity, together with bladder enlargement and sympathetic and sensory hyperinnervation (Schnegelsberg et al., 2010, Girard et al., 2011). At spinal cord level, NGF levels were also upregulated in SCI rats with bladder hyperactivity (Seki et al., 2002, Zvarova et al., 2004). Chronic intrathecal administration of NGF to normal rats also resulted in bladder hyperactivity, accompanied by hyperexcitability of bladder sensory afferents (Yoshimura et al., 2006). Changes in bladder function were accompanied by upregulation in the expression of TrkA and $p75^{NTR}$ in the bladder and in the neuronal pathways regulating bladder function (Qiao and Vizzard, 2002a, Qiao and Vizzard, 2002b, Murray et al., 2004, Qiao and Vizzard, 2005, Klinger et al., 2008, Klinger and Vizzard, 2008).

The effects of NGF blockade on bladder function have also been studied. In animals with cystitis, the effects of NGF have been inhibited by intravenous injection of REN1820, a recombinant protein able to sequester NGF (Hu et al., 2005), or following intravesical

l·ŘYƛyƛãú₪l·úƛ2y¯2ŧ l·y¯l·yúƛ-bDC l·yúƛō2Řè 2₪ l· ∃ŜySŜ₪l·f l·yúl·∃2yƛãű 2ŧ ¢₪ƫ ₪ŜÖŜLJú2₦ã óDdzŜ₦ƛ2ã Ŝú l·f¢Σ нллсΣ DdzŜ₦ƛ2ã Ŝú l·f¢Σ нллуú¢ Ly¯l·ff Ől·ãŜãΣ l· LJ₦2y2dzyÖŜŘ ƛY¯LJ₦2ØŜY¯Ŝyű 2ŧ ōfl·ŘŘŜ₦ ŧdzyÖúƛ2y¯ó l dz Ŝú l·f¢Σ нллрú l·yŘ ₪ŜŘdzÖúƛ2y¯ 2ŧ ØƛãÖŜ₦l·f LJl·ƛy¯ óDdzŜ₦ƛ2ã Ŝú l·f¢Σ нллсΣ DdzŜ₦ƛ2ã Ŝú l·f¢Σ нллуú ØŜ₦Ŝ 2ōãŜ₦ØŜŘ¢ [ƛƫŜØƛãŜΣ ƛyúNl·ØŜãƛÖl·f ƛyãúƛffl·úƛ2y¯ 2ŧ l·yúƛãŜyãŜ 2fƛ∃2ŘŜ2Èèy₪zÖtŜ2úƛŘŜãΣ ōŜŧ2₦Ŝ ƛyŘdzÖúƛ2y¯ 2ŧ ÖèãúúƛãΣ ōf2Öƫ̂ŜŘ bDC ãèyúKŜãƛã l·yŘ ₪ŜŘdzÖŜŘ ōfl·ŘŘŜ₦ KèLJŜ₦l·ÖúƛØƛúè ó¢èl·∃ƛ Ŝú l·f¢Σ нллсú¢ Ly¯ {/L ₦l·úãΣ ƛY¯Y¯dzy2yŜdzú₦l·fîl·úƛ2y¯ 2ŧ bDC l·ű úKŜ ãLJƛyl·f Ö2₪Ř fŜØŜf ₪ŜŘdzÖŜŘ ōfl·ŘŘŜ₦ KèLJŜ₦l·ÖúƛØƛúè l·yŘ ŘŜú₪dzã2₪-ãLJKƛyÖúŜ₦-ŘèããèySƛ∃ƛl· ó{Ŝƫƛ Ŝú l·f¢Σ нллнΣ {Ŝƫƛ Ŝú l·f¢Σ нллпú¢ {dz₦LJ₦ƛãƛyƎfèΣ ØKŜ₦Sl·ã ōf2Öƫ̂ƛyƎ úKŜ ƛyúŜ₦l·ÖúƛØƛúè¯ ãŜŜY¯ă ú2 ōŜ l·ŘØl·yúl·∃Ŝ2dzã ű2 ú₪Sl·ű ōfl·ŘŘŜ₦ KèLJŜ₦l·ÖúƛØƛúè¯ úKŜ ãl·Y¯Ŝ ól·ã yƎű 2ōãŜ₦ØŜŘ ŧ2ff2ØƛyƎ ƛyKƛōƛúƛ2y¯ 2ŧ LJтр$^{b¢w}$¢ LyúNl·ØŜãƛÖl·f ƛyãúƛffl·úƛ2y¯2ŧ t5флтулΣ ØKƛÖK ãLJŜÖƛŦƛÖl·ffè LJ₦ŜØŜYúă bDC ōƛyŘƛyƎ ű2 LJтр$^{b¢w}$Σ 2₪ 2ŧ l·y¯ l·yúƛō2Řè úl·₪∃Ŝúƛy∃ úKƛă b¢ă ₪ŜÖŜLJú2₪Σ ƛyÖ₪Sl·ãŜŘ Ø2ƛŘƛyƎ ŦW̃Sljdz̃SyÖè ō2úK ƛy¯ Ö2yú₪2f l·yŘ ƛy¯ l·yƛYl·fã ØƛúK Öèãúúƛã óYfƛyƎŜ₪ Ŝú l·f¢Σ нллуΣ YfƛyƎŜ₪ l·yŘ ±ƛÎÎl·₪ŘΣ нллуú¢ ¢KŜãŜ ŜŦŦŜÖúă ŦdzNúKŜ₪ ãú₪Sãä úKŜ ƛY¯LJ2₦úl·yÖŜ 2ŧ úKŜ ₪l·úƛ2 ¢₪ƫκLJтр$^{b¢w}$ ₪l·úKŜ₪ úKl·y¯ úKŜ ãƛyƎfŜ ŜÈLJ₪Sãäƛ2y¯ 2ŧ Ŝl·ÖK ₪ŜÖŜLJú2₪ ól dzl·yƎ l·yŘ wŜƛÖKl·₪ŘúΣ нллоú¢

¢KŜãŜ ŜÈLJŜ₪ƛYŜyúl·f ãűdzŘƛŜã l·ŘŘ₪Sãäƛy∃ úKŜ Yl·yƛLJdzfl·úƛ2y¯2ŧ bDC fŜØŜfã Kl·ØŜ ōŜŜy¯ŘƛŦŦƛÖdzfű ú2 ú₪l·yãfl·úŜ ƛyú2 úKŜ Öfƛy¯ÖãΦ {ûƛffΣ ƛy¯ l· ₪ŜÖŜyű ãűdzŘè ¢l·yŜÎdzYl·ō ól·ã dzãŜŘ ű2 ú₪Sl·ű LJl·úƛŜyúă ØƛúK . t{κL/¢ ¢l·yŜÎdzYl·ō fŜl·Ř ű2 l· ãfƛ∃Kű ₪ŜŘdzÖúƛ2y¯ƛy¯dz₪ƛyl·₪è Ŧ₪Sljdz̃SyÖè l·yŘ LJl·ƛy¯ōdzű ã2YŜ LJl·úƛŜyúă l·fã2 ₪ŜLJ2₪úŜŘ ãŜØŜ₪Ŝ ãƛŘŜ ŜŦŦŜÖúă ãdzÖK l·ã LJl·₪Sãúκ̃KŜãƛl·Σ KèLJŜ₪Sãúκ̃KŜãƛl· l·yŘ Y¯ƛ∃₪l·ySã ó9Øl·yã Ŝú l·f¢Σ нлммú¢ ¢l·yŜÎdzYl·ō Kl·ã l·fã2 ōŜŜy¯dzãŜŘ ƛy¯LJl·úƛŜyúă ØƛúK ÖK₪2yƛÖ LJ₪2ãúl·úƛúãkÖK₪2yƛÖ LJŜfØƛÖ LJl·ƛy¯ ãèyŘ₪2YŜΦ Ly¯ úKƛã ãűdzŘèΣ ƛY¯LJ₪ØŜY¯Ŝyű 2ŧ LJl·ƛy¯ l·yŘ dz₪∃ŜyÖè ØƛúK Yl·₪ƛyl·f l·yŘ úKŜ Y¯2ãű Ö2Y¯Y¯2y¯l·ŘØŜ₪ãŜ ŜØŜyúă ØŜ₪Ŝ LJl·₪Sãúκ̃KŜãƛl· l·yŘ l·₪úK₪l·fƎƛl· óbƛÖ]Ŝf Ŝú l·f¢Σ нлмнú¢

3. Brain-Derived Neurotrophic Factor (BDNF)

.5bC ƛã úKŜ Y¯2ãű dzōƛljdzƛû2dzã ySdz₪2ú₪2LJKƛy¯ ƛy¯ úKŜ ySØ₪2dzã ãèãúŜY¯ ótŜÎŜű Ŝú l·f¢Σ нллнÖú¢ .5bC Ö2yú₪ƛōdzúŜã ú2 úKŜ ŘƛŦŦŜ₪Syúƛl·úƛ2y¯2ŧ LJfl·Ö2ŘŜ-ŘŜ₪ØŜŘ ãŜyã2₪è ySdz₪2yã óŜ¢Ǝ úK2ãŜ ƛy¯úKŜ y2Ř2ãŜ Ǝl·yƎfƛ2yŭ l·yŘ ãdz₪ØƛØl·f 2ŧ l·Řdzfű ãŜyã2₪è ySdz₪2yã óō2úK LJfl·Ö2ŘŜ- l·yŘ ySdz₪l·f Ö₪Sãú-ŘŜ₪ØŜŘ ySdz₪2yãú ótŜÎŜű Ŝú l·f¢Σ нллнÖΣ tŜÎŜú l·yŘ aŎal·K2yΣ нллсú¢ !ÖÖ2₪Řƛy∃fèΣ ƛű ól·ã ãK2ó¯ úKl·ú .5bC ƛã ySÖŜããl·₪è Ŧ2₪ úKŜ ãdz₪ØƛØl·f 2ŧ ãf2ófè l·Řl·LJúƛy∃ Y¯ŜÖKl·y2₪ŜÖŜLJú2₪ã ó{!aú úKl·ú ƛyySØl·úŜ aŜ₪ƫŜf ÖŜffã ƛy¯ úKŜ ãƫƛy¯ó/l·₪₪2ff Ŝú l·f¢Σ мффуú l·yŘ .5bC ƫy2Öƫ2dzú Y¯ƛÖŜ LJ₪ŜãŜyú ₪ŜŘdzÖŜŘ ŜÈLJ₪Sãäƛ2y¯ 2ŧ !{L/нΣ l·y¯ ƛY¯LJ2₪úl·yú Y¯ŜÖKl·y2ú₪l·yãŘdzÖŜ₪ óaÖLfó₪l·úK Ŝú l·f¢Σ нллрú¢ Ly¯ l·ŘdzfúK22ŘΣ .5bC Kl·ã ōŜŜy¯ƛY¯LJfƛÖl·úŜŘ ƛy¯ úKŜ ₪Ŝ∃dzfl·úƛ2y¯2ŧ ySdz₪l·f ú₪l·yãY¯ƛãäƛ2y¯ l·yŘ ãèyl·LJúƛÖ LJfl·ãúƛÖƛúè ƛy¯ Y¯l·yê l·₪Sl·ã 2ŧ úKŜ ÖŜyú₪l·f ySØ2dzã ãèãúŜY¯Σ ƛyÖfdzŘƛy∃ úKŜ KƛLJLJ2Öl·Y¯LJdzãΣ Øƛãdzl·f Ö2₪úŜÈ l·yŘ ãLJƛyl·f Ö2₪Ř ó{ÖKƛyŘŜ₪ l·yŘ t22Σ нллΣ tŜÎŜű Ŝú l·f¢Σ нллнl·ú¢ a2ãű 2ŧ úKŜ ÖŜffdzfl·₪ l·Öúƛ2yã 2ŧ .5bC l·₪Ŝ YŜŘƛl·úŜŘ ōè úKŜ Kƛ∃K l·ŦŦƛyúè ₪ŜÖŜLJú2₪Σ ¢₪ƫ. óYl·LJfl·y¯ l·yŘ aƛffŜ₪Σ мффтúΣ

ØKƛÔK ƛă IꞏōdzyŘIꞏyΰ ŘdzNƛyƎ ŘŜØŜſ2LJYˇŜyΰ IꞏyŘ ØƛŘŜſè Řƛăΰ₪ƛōdzΰŜŘ ƛyˇ ΰKŜ LJŜ₪ƛLJKŜ₪Iꞏſ IꞏyŘ ŎŜyΰ₪Iꞏſ ySNØ2dzã ăèăΰŜY 2Ŧ IꞏŘdzſΰ IꞏyƛYꞏIꞏfã óIdzIꞏyƎ IꞏyŘ wŜƛÔKIꞏ₪ŘΰΣ нллмύΦ . 5bC ƛă Ô2yãΰƛΰdzΰƛ0ŜſèăèyΰKŜăƛÎŜŘ ōè Iꞏ ădzōLJ2LJdzfIꞏΰƛ2yˇ2Ŧ ăYˇIꞏff- IꞏyŘ YˇŜŘƛdzY-ăƛÎŜŘ LJŜLJΰƛŘŜ₪ƎƛÔ 5wD ySdz₪2yã óaŜ₪ƛƎKƛ Ŝΰ IꞏfΦΣ нллпύΣ ōdzΰ IꞏfãƨΣ ƛyˇ y2y-ySdz₪2yIꞏf ÔŜſfãΣ ƛyÔfdzŘƛyƎ ΰKŜ ŜLJƛΰKŜſƛIꞏ 2Ŧ ΰKŜ ōfIꞏŘŘŜ₪Σ fdzyƎ IꞏyŘ Ô2ſ2yˇó[2YˇIꞏΰÎăÔK Ŝΰ IꞏfΦΣ мфффύΦ

. 5bC ƛă Ô2yãƛŘŜ₪ŜŘ Iꞏ ÔŜyΰ₪Iꞏſ YˇƨŘdzfIꞏΰƨ₪ Iꞏăƛΰ ÔIꞏyˇōŜ IꞏyΰŜ₪2Ǝ₪IꞏŘŜſè Ú₪IꞏyãLJƨ₪ΰŜŘ ōè ăŜyãƨ₪è ySdz₪2yã ΰƨ KŜ ăLJƛyIꞏf Ô2₪ŘΣ ØKŜ₪Ŝ ƛΰ ƛă ₪ŜſŜIꞏăŜŘ ƛyˇŘ2₪ăIꞏf K2₪yˇƛyˇfIꞏYˇƛyIꞏŜ L IꞏyŘ LL dzLJ2yˇyƨÈƛ2dzã LJŜ₪ƛLJKŜ₪Iꞏſ ăΰƛYˇdzfIꞏΰƛ2yˇ ó½K2dz IꞏyŘ wdzăKΣ мффсύΦ LyΰŜ₪ŜăΰƛyƎfèΣ . 5bC ŜÈLJ₪Ŝăăƛƨyˇ ƛyˇ ¢₪ʅ!-ŜÈLJ₪ŜăăƛyƎ ăŜyãƨ₪è ySdz₪2yã ƛă ₪Ŝ3dzfIꞏΰŜŘ ƛyˇ Iꞏ bDC-ŘŜLJŜyŘŜyΰ YˇIꞏyŸŜ₪ óaƛÔKIꞏŜſ Ŝΰ IꞏfΦΣ мффтΣ tŜÎŜΰ ŜΰIꞏfΦΣ нллнÔΣ aŜ₪ƛƎKƛ ŜΰIꞏfΦΣ нллпΣ aŜ₪ƛƎKƛ ŜΰIꞏfΦΣ нллуύΦ Lΰƛă Ô2yΰIꞏƛyŜŘ ƛyˇfIꞏ₪ƎŜ ŘŜyãŜ Ô2₪Ŝ ăèyIꞏLJΰƛÔ ØŜăƛÔfŜă ó5/±ăύ ƛyˇ ΰŜ₪YˇƛyIꞏfã 2Ŧ LJNƛYˇIꞏ₪è IꞏŦŦŜ₪Ŝyΰ ŦƛōŜ₪ăΣ ØKŜ₪Ŝ ƛΰ ƛă Ô2-ăΰ2₪ŜŘ ØƛΰK {dzōăΰIꞏyÔŜ t IꞏyŘ /IꞏfÔƛΰ2yƛyˇ DŜySŜ wŜſIꞏΰŜŘ LJŜLJΰƛŘŜ ó/Dwtύ óaŜ₪ƛƎKƛ ŜΰIꞏfΦΣ нллпΣ {IꞏſƨΣ ŜΰIꞏfΦΣ нллтύΦ hyÔŜ ₪ŜſŜIꞏăŜŘ ƛyˇΰKŜ Ř2₪ăIꞏf K2₪yΣ . 5bC ōƛyŘă ΰ2 ¢₪ʅ.Σ LJN₪ŜăŜyΰ ƛyˇLJN₪ƛYˇIꞏ₪è IꞏŦŦŜ₪ŜyΰăIꞏyŘ LJ2ăΰăèyIꞏLJΰƛÔ ySdz₪2yIꞏf LJN₪2ŦƛſŜă ótŜÎŜΰ ŜΰIꞏfΦΣ нллнōΣ {IꞏſƨΣ ŜΰIꞏfΦΣ нллрΣ aŜ₪ƛƎKƛ ŜΰIꞏfΦΣ нллуύΣ IꞏyŘ ƛyŘdzÔŜă LJ2ΰŜyΰƛIꞏΰƛ2yˇ2Ŧ ƎfdzΰIꞏYˇIꞏΰŜ₪ƎƛÔ ySdz₪2ΰ₪IꞏyãYˇăăƛ2yˇØƛIꞏ Ř2øyãΰ₪ŜIꞏYˇ IꞏÔΰƛØIꞏΰƛ2yˇ2Ŧ ΰKŜ 9wY IꞏyŘ tY/ LJIꞏΰKøIꞏèă ótŜÎŜΰ ŜΰIꞏfΦΣ нллнōΣ {flꞏÔʅ IꞏyŘ ¢K2YˇLJă2yΣ нллнΣ {flꞏÔʅ ŜΰIꞏfΦΣ нллпΣ {flꞏÔʅ ŜΰIꞏfΦΣ нллрύΦ

3.1 BDNF as a pain modulator

LyˇΰKŜ IꞏŘdzfΰΣ . 5bC ƛă IꞏyˇƛYLJ2₪ΰIꞏyΰ Y2ŘdzfIꞏΰƨ₪ 2Ŧ ăŜyãƨ₪è Ú₪IꞏyãYˇƛăăƛ2yΣ LJfIꞏèƛyƎ IꞏLJIꞏ₪ΰƛÔdzfIꞏ₪ſè ƛYˇLJ2₪ΰIꞏyΰ ₪2fŜ ƛyˇ₪ŜƎdzfIꞏΰƛyƎ y2ÔƛÔŜLJΰƛØŜ LJIꞏΰKøIꞏèăΦ [ŜØŜſă 2Ŧ . 5bC Iꞏ₪Ŝ dzLJN₪ŜƎdzfIꞏΰŜŘ ƛyˇKdzYˇIꞏyˇÔK₪2yƛÔ LJIꞏƛyŦdzf LJIꞏΰK2f2ƎƛŜă ădzÔK Iꞏă Iꞏ₪ΰK₪ΰƛă óŘŜſ t2₪ΰƨ2 Ŝΰ IꞏfΦΣ нллсΣ D₪ƛYˇăK2fYˇ Ŝΰ IꞏfΦΣ нллуIꞏΣ D₪ƛYˇăK2fYˇ Ŝΰ IꞏfΦΣ нллуōύΦ LyˇN₪IꞏΰăΣ ƛyΰ₪IꞏLJfIꞏyΰIꞏ₪ IꞏŘYˇƛyIăΰ₪Iꞏΰƛ2yˇ2Ŧ . 5bC ƛyŘdzÔŜă Ú₪IꞏyãŜyΰ ΰKŜ₪YˇIꞏf KèLJŜ₪IꞏfƎŜăƛIꞏ LJ2ăăƛōfè ōè ăøƛŦΰfè ăŜyãƛΰƛÎƛyƎ LJŜ₪ŜLJKŜ₪Iꞏf ăŜyãƨ₪è ySdz₪2yã ØƛIꞏ ¢₪ʅ.₪ŜÔŜLJΰƨ₪ ó{Kdz Ŝΰ IꞏfΦΣ мфффύΦ 5ŜſƛØŜ₪è 2Ŧ . 5bC ŘƛΰŜÔΰfè ΰ2 5wDă ÔIꞏdzăŜŘ YˇŜÔKIꞏyƛÔIꞏf Iꞏff2Řèyƛꞏ ó½K2dz Ŝΰ IꞏfΦΣ нлллύ IꞏyŘ ƛyΰ₪IꞏΰKŜÔIꞏf ŘŜſƛØŜ₪è 2Ŧ . 5bC ƛyŘdzÔŜŘ LJ₪2YˇƛySyΰ 2Ŧ Ô-C2ă ŜÈLJ₪Ŝăăƛƨyˇ ó¢K2YˇLJă2yˇ Ŝΰ IꞏfΦΣ мфффύΣ Iꞏyˇ ŜăΰƛIꞏōfƛăKŜŘ ăLJƛyIꞏf YˇIꞏ₪ʅŜ₪ 2Ŧ yƨÈƛ2dzã ƛyΰLJdzΰ ó/2ƎƎŜăKIꞏffΣ нллрύΦ LyˇN₪IꞏΰăΣ ădzōYˇƛΰΰŜŘ ΰ2 ăÔƛIꞏΰƛÔ ySNØŜ ΰ₪IꞏyãŜÔΰƛ2yΣ Iꞏff2Řèyƛꞏ ØƛIꞏăΣ IꞏfăƨΣ ŘŜLJŜyŘŜyΰ 2yˇ . 5bC Iꞏă ƛΰ ØƛIꞏă IꞏΰΰŜyΩdzIꞏΰŜŘ ōè ŘƛΰŜÔΰ IꞏLJLJfƛÔIꞏΰƛ2yˇΰ2 ƛyƨdz₪ŜŘ 5wDă 2Ŧ IꞏyˇIꞏyΰƛ-. 5bC IꞏyΰƛōƨŘè ó½K2dz Ŝΰ IꞏfΦΣ нлллύΦ [ʅŜØƛăŜΣ ă2YˇIꞏΰƛÔ KèLJŜ₪IꞏfƎŜăƛIꞏ ÔIꞏdzăŜŘ ōè ySNØŜ fƛƎIꞏΰƛ2yˇ ØƛIꞏăΣ IꞏfăƨΣ ₪ŜØŜ₪ΰŜŘ ōè IꞏyˇIꞏyΰƛ-. 5bC IꞏyΰƛōƨŘè óCdzʅdz2ʅIꞏ Ŝΰ IꞏfΦΣ нллмύΦ wIꞏΰă ₪ŜÔŜƛØƛyƎ ƛyΰ₪IꞏŘŜ₪YˇIꞏf ƛyƨŜÔΰƛ2yˇ 2Ŧ Ŧ2₪YˇIꞏfƛyˇ óYŜ₪₪ Ŝΰ IꞏfΦΣ мфффΣ ¢K2YˇLJă2yˇ Ŝΰ IꞏfΦΣ мфффύ 2₪ ÔIꞏ₪₪IꞏƎŜŜyIꞏyˇ óD₪2ΰK IꞏyŘ ! Iꞏy2yãŜyΣ нллнύ IꞏfăƨΣ ŘŜØŜſ2LJŜŘ . 5bC-ŘŜLJŜyŘŜyΰ ÔdzΰIꞏyŜ2dzã KèLJŜ₪ăŜyãƛΰƛØƛΰèΦ ¢Kƛă LJIꞏƛyˇ ōŜKIꞏØƛ2dz₪ ØIꞏă ₪ŜØŜ₪ΰŜŘ IꞏŦΰŜ₪

ƛyú₪I·úKŜÖI·f I·ŘYƛyƛãú₪I·úƛ2y2Ŧ I· ãŜIjdzŜãúŜ₪ƛyƎ I·yúƛō2ŘèΣ ¢₪ʅ .-LƎD óYŜ₪₪ Ŝű I·f℗Σ мффффΣ ¢K2YLJă2y Ŝű I·f℗Σ мфффú Σ 2₪ I·yúƛãŜyãŜ 2fƛƎ2ŘŜ2Ëèyd zÖfŜ2úƛŘŜã óD₪2űK I·yŘ ! I·y2yãŜyΣ нллнú℗

!fűK2dzƎK úKŜ ₪2fŜ 2Ŧ . 5bC ƛY ă2YI·ûƛÖ LJI·ƛY ƛã ǿŜft Ř2ÖdzYŜyűŜŘ ótŜÎŜű Ŝű I·f℗Σ нллнÖΣ aŜ₪ƛƎKƛ Ŝű I·f℗Σ нллпΣ aŜ₪ƛƎKƛ Ŝű I·f℗Σ нллуúΣ ₪ŜLJ2₪úã ŘŜãÖ₪ƛōlyƎ úKŜ Ö2yú₪ƛōdzûƛ2y 2Ŧ . 5bC ú2 ØƛãÖŜ₪I·f LJI·ƛY I·₪Ŝ Y2₪Ŝ ₪ŜÖŜyű I·yŘ 2yfè ŜËƛãű ƛY I· ãYI·ff ydzYōŜ₪℗ LY KdzYI·yãΣ I·y dzLJ₪ŜƎdzfI·ûƛ2y 2Ŧ . 5bC ƛY úKŜ ƛyŦfI·YŜŘ LJI·yÖ₪ŜI·ãΣ LJ₪ŜãŜyű ƛY yŜ₪ØŜ ŦƛōŜ₪ãΣ ƎfI·yŘ Ř dzÖű ÖŜftãΣ ǿI·ã 2ōãŜ₪ØŜŘ I·yŘ Ö2₪₪ŜfI·ûŜŘ ǿƛúK ãŜØŜ₪Ŝ LJI·ƛY ó½Kdz Ŝű I·f℗Σ нллмú℗ ! ₪2fŜ Ŧ2₪ . 5bC ƛY ƛ₪₪ƛúI·ōfŜ ō2ǿŜf ŘƛãŜI·ãŜ KI·ã I·fã2 ōŜŜY ₪ŜÖŜyűfè ãdzƎƎŜãúŜŘΣ I·ã . 5bC ŜËLJ₪Ŝããƛ2y ƛY Ö2f2y YdzÖ2ãI· ǿI·ã dzLJ₪ŜƎdzfI·ûŜŘ I·yŘ I·ãã2ÖƛI·ûŜŘ ǿƛúK ãú₪2yƎ I·ōŘ2YƛyI·f LJI·ƛY ƛY LJI·úƛŜyűã ǿƛúK ƛ₪₪ƛúI·ōfŜ ō2ǿŜf ãèyŘ₪2YŜ ó¸dz Ŝű I·f℗Σ нлмнú℗ LyűŜ₪Ŝãúƛy Ǝfè Σ .5bC KŜúŜ₪2Îè Ǝ2dzã ŘŜŦƛÖƛŜyű YλÖŜ ǿƛúK Ö2fƛúƛã LJ₪ŜãŜyűŜŘ ₪ŜŘdzÖŜŘ Ö2f2yƛÖ KèLJŜ₪ãŜyãƛúƛØƛúè I·yŘ ōfI·ŘŘŜ₪ KèLJŜ₪I·ÖúƛØƛúè ƛY Ö2YLJI·₪ƛă2y ǿƛúK ǿƛfŘ úèLJŜ fƛúúŜ₪YI·úŜã ó¸I·yƎ Ŝű I·f℗Σ нлмлú℗

¢KŜ YŜÖKI·yƛãYă 2Ŧ . 5bC-YŜŘƛI·ûŜŘ LJI·ƛY I·₪Ŝ ₪ŜfI·ûŜŘ ǿƛúK Ř2óyãú₪ŜI·Y I·ÖúƛØI·úƛ2y 2Ŧ úKŜ 9wY LJI·úKǿI·è I·ú úKŜ ãLJƛyI·f Ö2₪Ř fŜØŜf óYŜ₪₪ Ŝű I·f℗Σ мфффΣ tŜÎŜű Ŝű I·f℗Σ нллнōΣ [ŜØŜ₪ Ŝű I·f℗Σ нллоōúΣ ₪I·LJƛŘfè I·ÖúƛØI·ûŜŘ Ŧ2ff2óƛyƎ ă2YI·úƛÖ I·yŘ ØƛãÖŜ₪I·f yŽÈƛ2dzã ãúƛYdzfI·ûƛ2y óWƛ Ŝű I·f℗Σ мфффΣ YI·₪ƛY Ŝű I·f℗Σ нллмΣ DI·fI·y Ŝű I·f℗Σ нллнΣ DI·fI·y Ŝű I·f℗Σ нллоΣ YI·óI·ãI·ʅƛ Ŝű I·f℗Σ нллпΣ /₪dzÎ Ŝű I·f℗Σ нллpI·ú℗ 9wY ƛyKƛōúƛ2yΣ I·ÖKƛŜØŜŘ ōè ƛyú₪I·ØŜy2dzã 2₪ ƛyú₪I·úKŜÖI·f I·ŘYƛyƛãú₪I·úƛ2y 2Ŧ 9wY ƛyKƛōúƛ2₪ãΣ KI·ã ōŜŜy ădzÖÖŜãăŦdzftfè dzãŜŘ ú2 ₪ŜŘdzÖŜ LJI·ƛy 2Ŧ ă2YI·úƛÖ I·yŘ ØƛãÖŜ₪I·f 2₪ƛƎƛy óWƛ Ŝű I·f℗Σ нллнI·Σ DI·fI·y Ŝű I·f℗Σ нллоΣ !Řó I·yʅI·₪ Ŝű I·f℗Σ нллпΣ YI·óI·ãI·ʅƛ Ŝű I·f℗Σ нллпΣ /₪dzÎ Ŝű I·f℗Σ нллpI·Σ /₪dzÎ Ŝű I·f℗Σ нллpōΣ ¸dz I·yŘ /KŜyΣ нллрΣ tI·yƎ Ŝű I·f℗Σ нллуú℗

3.2 BDNF in LUT

[ƛúúfŜ ƛă ʅy2óy I·ō2dzű úKŜ ŜËLJ₪Ŝăãƛ2y 2Ŧ . 5bC ƛY úKŜ ōfI·ŘŘŜ₪Σ ō2úK ŘdzƛyƎ ŘŜØŜf2LJYŜyű I·yŘ ƛY I·ŘdzfűK22ŘΣ 2₪ I· LJ2ûŜyűƛI·f ₪2fŜ 2Ŧ . 5bC ƛY ōfI·ŘŘŜ₪ ŦdzyÖúƛ2y ō2úK ƛY y2₪YI·f I·yŘ ƛY LJI·úK2f2ƎƛÖI·f Ö2yŘƛúƛ2yã℗ LY KdzYI·yãΣ úKŜ LJ₪ŜãŜyÖŜ 2Ŧ . 5bC I·yŘ ƛűă KƛƎK I·ŦŦƛyúè ₪ŜÖŜLJű2₪ ¢₪ʅ . KI·ã ōŜŜy ŘŜăÖ₪ƛōŜŘ ƛY ōfI·ŘŘŜ₪ ÖI·yÖŜ₪ ÖŜftă óIdzI·yƎ Ŝű I·f℗Σ нлмлΣ [I·ƛ Ŝű I·f℗Σ нлмлú ōdzű úKŜƛ₪ ŜËLJ₪Ŝăãƛ2yƛY KŜI·fúKè úƛăădzŜ KI·ã y2ű ōŜŜy ăúdzŘƛŜŘ℗ LY úKŜ ₪I·úΣ úKŜ YI·ż2₪ ă2dz₪ÖŜ 2Ŧ . 5bC ăŜŜYă ű2 ōŜ úKŜ dz₪2úKŜfƛdzY ō2úK ƛY úKŜ ŜYō₪è2 I·yŘ ƛY I·Řdzfű ó[2YYI·úÎăÖK Ŝű I·f℗Σ мфффΣ [2YYI·úÎăÖK Ŝű I·f℗Σ нллрú℗ LY ₪I·úăΣ ƛű KI·ã ōŜŜy LJ₪2LJ2ăŜŘ úKI·ú úKŜ Řèyl·YλÖ LJI·úúŜ₪Y 2Ŧ . 5bC ŜËLJ₪Ŝăãƛ2y ŘdzƛyƎ ŘŜØŜf2LJYŜyúΣ ǿƛúK ŜËLJ₪Ŝăãƛ2y LJŜI·ʅă I·ű tp I·yŘ tмлΣ YI·è ₪ŜŦfŜÖú úKŜ ₪ŜLJfI·ÔŜYŜyű 2Ŧ úKŜ ƛYYI·ûdz₪Ŝ ăLJƛyI·f YλÖúdz₪ƛúƛ2y ₪ŜŦfŜË ōè úKŜ ăLJƛy2ōdzfō2ăLJƛyI·f ₪ŜŦfŜË LJI·úKǿI·èΣ ǿKλÖK Ǝ2ØŜ₪y ãYλÖúdz₪ƛúƛ2y ƛY I·Řdzfű I·yƛYI·fã óC2ǿfŜ₪ Ŝű I·f℗Σ нллуú℗ CƛyI·ffèΣ I·fűK2dzƎK úKŜ LJ₪ŜÖƛãŜ ă2dz₪ÖŜ ƛă èŜű ű2 ōŜ Ŧdzffè Ŝăű I·ōfƛăKŜŘΣ .5bC ÖI·y I·fã2 ōŜ Ŧ2dzyŘ ƛY ăYI·ff Ijdz I·yúƛúƛŜă ƛY úKŜ dz₪ƛyŜ 2Ŧ KŜI·fűKè ƛyŘƛØƛŘdzI·fã ó!yúdzyŜă-[2LJŜă Ŝű I·f℗Σ нлмоú℗

3.2.1 BDNF and bladder dysfunction

.ŜŎI٠dzăŜ ƛy˜ ăSyã2₦è ySdz₦2yã .5bC ŜÈLJ₦Ŝăăƛ2y˜ ƛă dzLJ₦ŜƎdzfI٠ŭŜŘ ₮2ff2ǿƛyƎ bDC ŜÈLJ2ădz₦Ŝ óaƛŎKI٠Ŝf Ŝŭ I٠f₱Σ мффтΣ YŜ₦₦ Ŝŭ I٠f₱Σ мфффúΣ ƛŭ ƛă 2Ŧ ƛyŭŜ₦Ŝăŭ ŭ2 I٠ăăŜăă ŭKŜ ₦2fŜ 2Ŧ .5bC ƛy˜ōfI٠ŘŘŜ₦ Řèă₮dzyŎŭƛ2y˜I٠ăă2ŎƛI٠ŭŜŘ ǿƛŭK KƛƎK bDC fŜØŜfă℗ LyŘŜŜŘΣ ƛy˜h! . I٠yŘ . t{ĸL/ LJI٠ŭŜyŭă2 dz₦ƛyI٠₦è .5bC ǿI٠ă ŜfŜØI٠ŭŜŘ I٠yŘ Ŏ2₦₦ŜfI٠ŭŜŘ ǿƛŭK dz₦ƎŜyŎèΣ Ŧ₦ŜIjdzŜyŎè I٠yŘ LJI٠ƛy˜ótƛyŭ2 Ŝŭ I٠f₱Σ нлмlōΣ !yŭdzySă-[2LJŜă Ŝŭ I٠f₱Σ нлмoú℗ C2ff2ǿƛyƎ ădzŎŎŜăăŦdzf ú₦ŜI٠ŭY˜ŜyŭΣ dz₦ƛyI٠₦è .5bC ǿI٠ă Ř2ǿy₦ŜƎdzfI٠ŭŜŘ ótƛyŭ2 Ŝŭ I٠f₱Σ нлмlōΣ !yŭdzySă-[2LJŜă Ŝŭ I٠f₱Σ нлмoú

Ly˜₦I٠ŭăΣ ŭKŜ ŜÈLJ₦Ŝăăƛ2y˜ .5bC Y˜wb! ƛy˜ŭKŜ ōfI٠ŘŘŜ₦ ƛă ƛyŎ₦ŜI٠ăŜŘ Ŧ2ff2ǿƛyƎ Ŏèăŭƛŭƛă ƛyŘdzŎŜŘ ōè ƛyŭ₦I٠LJŜ₦ƛú2yŜI٠f ƛy?ŜŎŭƛ2y˜ 2Ŧ ŎèŎf2LJK2ăLJKI٠Y˜ƛŘŜ ó/¸tú ó±ƛÎÎI٠₦ŘΣ нлллú 2₦ ƛyãŭƛffI٠ŭƛ2y˜ 2Ŧ ŭdz₦LJŜyŭƛyŜ óhŘŘƛI٠K Ŝŭ I٠f₱Σ мффyú℗ LyŭŜ₦ŜăŭƛyƎfèΣ .5bC ŜÈLJ₦Ŝăăƛ2y˜ƛă I٠fă2 dzLJ₦ŜƎdzfI٠ŭŜŘ ƛy˜ōfI٠ŘŘŜ₦ ăSyã2₦è I٠Ŧ₮Ŝ₦Syŭă ƛy˜ ₦I٠ŭă ǿƛŭK Ŏ2fƛŭƛă ƛyŘdzŎŜŘ ōè ƛyŭ₦I٠Ŏ2f2yƛŎ ƛ₦₦ƛƎI٠ŭƛ2y˜ ǿƛŭK ŭ₦ƛ-yŭ₦2ōŜyÎŜyŜ ădzfŦ2yƛŎ I٠ŎƛŘ ó¢b.{ú óvƛI٠2 I٠yŘ D₦ƛŘŜ₦Σ нллтúΣ ădzƎƎŜăŭƛyƎ ŭKI٠ŭ .5bC Y˜I٠è LJI٠₦ŭƛŎƛLJI٠ŭŜ ƛy˜Ŏ2f2y˜ú2-ōfI٠ŘŘŜ₦ Ŏ₦2ăă-ăŜyãƛŭƛÎI٠ŭƛ2y℗ Ly˜₦I٠ŭă ǿƛŭK ySdz₦2ƎŜyƛŎ ŘŜú₦dzã2₦ 2ØŜ₦I٠ŎŭƛØƛúè ób5hú ₦Ŝădzfŭƛy Ŧ₦2Y {/LΣ .5bC Y˜wb! ƛă I٠fă2 dzLJ₦ŜƎdzfI٠ŭŜŘ ƛy˜ŭKŜ ōfI٠ŘŘŜ₦ ó±ƛÎÎI٠₦ŘΣ нллúφ ¢KŜ Ŏ2yŎŜyú₦I٠ŭƛ2y˜ 2Ŧ ŭKŜ LJ₦2ŭŜƛy˜ƛă I٠fă2 ƛyŎ₦ŜI٠ăŜŘ ƛy˜ŭKŜ [cк{м ăLJƛyI٠f Ŏ2₦Ř ăŜƎY˜Syŭă ó½ØI٠₦2ØI· Ŝŭ I٠f₱Σ ннллпú℗ !ŎŎ2₦ŘƛyƎfèΣ Ŧ2ff2ǿƛyƎ Ŏèăŭƛŭƛă I٠yŘ {/LΣ ŭKŜ₦Ŝ ƛă ƛyŎ₦ŜI٠ăŜŘ ŜÈLJ₦Ŝăăƛ2y˜I٠yŘ I٠ŎŭƛØI٠ŭƛ2y˜2Ŧ ŭKŜ ¢₦ℐ. ₦ŜŎŜLJŭ2₦ ƛy˜ōfI٠ŘŘŜ₦ ăSyã2₦è ySdz₦2yã óvƛI٠2 I٠yŘ ±ƛÎÎI٠₦ŘΣ нллнI٠Σ vƛI٠2 I٠yŘ ±ƛÎÎI٠₦ŘΣ нллнōúΣ ǿKƛŎK ădzƎƎŜăŭă ŭKI٠ŭ .5bC ƛă dzLJ-ŭI٠ℐŜy˜ōè ōfI٠ŘŘŜ₦ I٠Ŧ₮Ŝ₦Syŭă℗

4. Goals

CN2Y ΰKŜ LJNŜăŜyΰ NŜØƛŜøΣ ƛΰ ōŜŎ2YŜă ŜØƛŘŜyΰ ΰKI·ΰ Y I·yȅ ƛăădzŜă NŜƎI·NŘƛyƎ ΰKŜ ƛY LJ2NΰIıyŎŜ 2Ŧ ySdzN2ΰN2LJKƛyã ƛy ΰKŜ ōfI·ŘŘŜN NŜY I·ƛy dzyNŜă2fØŜŘΦ LyŘŜŜŘΣ bDC ƛă ŎfŜI·Nŧȅ ΰKŜ Y2ăΰ øŜft ăΰdzŘƛŜŘ ySdzN2ΰN2LJKƛyã ƛy ΰKŜ [¦¢ øƛύK ſƛΰΰŧŜ ŘI·ΰI· I·ØI·ƛfI·ōfŜ I·ō2dzΰ 2ΰKŜN b¢ăΦ Ly NŜăLJŜŎΰ ΰ2 bDCΣ *in vitro* I·ăăI·èă I·yŘ ăΰdzŘƛŜă Ŧ2ŎdzăƛyƎ 2y ă2YI·ΰƛŎ ăŜyãI·ΰƛ2y KI·ØŜ ŜăΰI·ōfƛăKŜŘ Ř2óyãΰNŜI·Y ΰI·NƎŜΰă I·yŘ ŜŦŦŜŎΰ2Nă 2Ŧ bDC ōdzΰ ƛΰ ƛă dzyŎfŜI·N ƛŦ ΰKƛă ˥y2øfŜŘƎŜ ŎI·y ōŜ ŜÈLJI·yŘŜŘ ΰ2 NŜƎdzfI·ΰƛ2y 2Ŧ ōfI·ŘŘŜN ăŜyãI·ΰƛ2y I·yŘ ŦdzyŎΰƛ2yΦ a 2NŜ2ØŜNΣ ΰKŜNŜ ƛă Ŏ2yãƛŘŜNI·ōfè fŜää ŘI·ΰI· NŜƎI·NŘƛyƎ ΰKŜ ƛY LJ2NΰIıyŎŜ 2Ŧ 2ΰKŜN ySdzN2ΰN2LJKƛyã ƛy ōfI·ŘŘŜN ŦdzyŎΰƛ2yΣ Y2ăΰ y2ΰI·ōfè . 5bCΣ 2yŜ 2Ŧ ΰKŜ Y2ăΰ I·ōdzyŘI·yΰ b¢ăΦ ¢KŜNŜŦ2NŜΣ ΰKŜ LJNŜăŜyΰ øNI ˥ KI·Ř ΰKNŜŜ Y I·ƛY ƎƗI·fãΣ ΰKŜ ŦANăΰ ΰøƗ NŜfI·ΰŜŘ øƛύK . 5bC I·yŘ ΰKŜ fI·ăΰ 2yŜ Ŧ2ŎdzăƛyƎ 2y bDCΦ ² Ŝ LJN2LJ2ăŜŘ ΰ2 I·ŘŘNŜăă ΰKŜ Ŧ2ff2øƛyƎ LJ2ƛyΰăY

- MΦ ¢2 ăΰdzŘè ΰKŜ N2fŜ 2Ŧ . 5bC ƛy ƛyŦfI·Y Y I·ΰƛ2y-ƛyŘdzŎŜŘ ōfI·ŘŘŜN Řèă₮dzyŎΰƛ2y I·yŘ LJI·ƛyΦ
- HΦ ¢2 ăΰdzŘè ΰKŜ N2fŜ 2Ŧ . 5bC ƛy ŜăΰI·ōfăKƛyƎ I·yŘ Y I·ƛyΰŜyI·yŎŜ 2Ŧ b5hΦ
- OΦ ¢2 ŜØI·fdzI·ΰŜ ƛŦ ΰKŜ ƛyΰŜNLJfI·è ōŜΰøŜŜy bDC I·yŘ ¢wt±MΣ ƛY LJ2NΰIıyΰ Ŧ2N ă2YI·ΰƛŎ LJI·ƛyΣ ƛă I·fă2 2LJŜNI·ΰƛyƎ ŘdzNƛyƎ ōfI·ŘŘŜN KèLJŜNI·ŎΰƛØƛΰè I·yŘ LJI·ƛy ƛy ΰKŜ f2øŜN I·ōŘ2YŜy I·yŘ KƛyŘLJI·øăΦ Ly I·yIY I·fă øƛύK Ŏèăΰƛΰƛă ƛyŘdzŎŜŘ ōè ƛyΰNI·LJŜNƛΰ2ySI·f ƛy?ŜŎΰƛ2y 2Ŧ / ¸ tΣ ōfI·ŘŘŜN I·yŘ ăLJƛyI·f Ŏ2NŘ . 5bC fŜØŜfă øŜNŜ dzLJNŜƎdzfI·ΰŜŘΦ LyΰNI·ØŜy2dză I·yŘ ƛyΰNI·ΰKŜŎI·f I·ŘY ƛyƛăΰNI·ΰƛ2y 2Ŧ ¢N ˥ .-LƎ„Σ I· NŜŎ2YōƛyI·yΰ LJN2ΰŜƛy ΰKI·ΰ ySdzΰNI·fΛÎŜă . 5bCΣ ŜŦŦŜŎΰƛØŜfè ƛYLJN2ØŜŘ ōfI·ŘŘŜN Řèă₮dzyŎΰƛ2y I·yŘ LJI·ƛy øI·ă I·ō2făKŜŘΦ ¢KŜăŜ NŜădzfΰă ƛyŘƛŎI·ΰŜ ΰKI·ΰ . 5bC ăă I·y ƛYLJ2NΰIıyΰ Y ŜŘƛI·ΰ2N ƛy ØăŎŜNI·f LJI·ƛy I·yŘ ōfI·ŘŘŜN Řèă₮dzyŎΰƛ2y ŘdzNƛyƎ ƛyƎfI·Y Y I·ΰƛ2y óCNƛI·ă Ŝΰ I·fΦΣ нлмнōΣ CNƛI·ă Ŝΰ I·fΦΣ нлмоύΦ

¢KŜ N2fŜ 2Ŧ . 5bC ƛy ΰKŜ ŜY ŜNƎŜyŎŜ I·yŘ Y I·ƛyΰŜyI·yŎŜ 2Ŧ b5h ƛy I·yƛY I·fă øƛύK I· Ŏ2YLJfŜΰŜ ăLJƛyI·f Ŏ2NŘ ΰNI·yãŜŎΰƛ2y øI·ă I·fă2 I·ŘŘNŜăăŜŘΦ wŜădzfΰă ăK2ø I· ΰƛY Ŝ-ŘŜLJŜyŘŜyΰ dzLJNŜƎdzfI·ΰƛ2y ƛy ăLJƛyI·f . 5bCΣ I·ŎŎ2YLJI·yƛŜŘ ōè ΰKŜ Ŝăΰ I·ōfăKƛyƎ 2Ŧ b5hΦ . 5bC ăŜljdzŜăΰNI·ΰƛ2y NŜădzfΰŜŘ ƛy I·y ŜI·NfƛŜN I·LJLJŜI·NI·yŎŜ 2Ŧ b5h ΰ2ƎŜΰKŜN øƛύK LJNŜY I·ΰdzNŜ ăLJN2dzΰƛyƎ 2Ŧ ōfI·ŘŘŜN I·Ŧ₮ŜNŜyΰΣ Ŏ2yŦANY ŜŘ ōè ŎŜff ŎdzfΰdzNŜ I·ăăI·èăΦ hy ΰKŜ Ŏ2yΰNI·NȅΣ ƛyΰNI·ΰKŜŎI·f . 5bC I·ŘY ƛyƛăΰNI·ΰƛ2yΣ ƛyƛΰƛI·ΰŜŘ ƛY Y ŜŘƛI·ΰŜfè I·Ŧ₮ŜN {/LΣ ŘŜfI·èă ΰKŜ ŜY ŜNƎŜyŎŜ 2Ŧ b5hΦ ¢KŜăŜ NŜădzfΰă ƛyŘƛŎI·ΰŜ ΰKI·ΰΣ Ŧ2ff2øƛyƎ {/LΣ . 5bC KI·ă I·LJN2ΰŜŎΰƛØŜ N2fŜ Ŧ2N ōfI·ŘŘŜN ŦdzyŎΰƛ2yΣ LJ2ăăƛōfè ōè ŘŜLJNŜăăƛyƎ ΰKŜ ŜăΰI·ōfăKY Ŝyΰ 2Ŧ b5h ŘdzNƛyƎ ΰKŜ yI·ΰdzNI·f Ŏ2dzNăŜ 2Ŧ ΰKŜ LJI·ΰK2f2ƎèΦ ¢Kƛă Y I·yΰdzăŎNƛLJΰ KI·ă I·fNŜI·Ře ōŜŜy ădzōYƛΰΰŜŘΦ

Ly˙úKŜ f˙ăű LJ˙LJŜ₦Σ úKŜ ƛyűŜ₦˙Őúƛ2y˙ōŜűøŜŜy˙¢wt±м ˙yŘ bDC ø˙ă ƛyØŜăűƛ∃˙úŜŘ ƛy˙dzăƛy∃ øƛfŘ-ûèLJŜ ó ² ¢ú ˙yŘ ¢wt±м ˥y2Ŏ˥-2dzű óYhú Y˙ƛŐŜΦ ! yΛY˙fă øŜ₦Ŝ ădzōY˙ƛúűŜŘ ű2 LJ₦2f2y∃ŜŘ ŜÈLJ2ădz₦Ŝ ű2 bDC ˙yŘ úKŜ ŜŦŦŜŐúă 2y˙ōf˙ŘŘŜ₦ ₦ŜŦfŜÈ ˙ŐúƛØƛúè ˙yŘ ōf˙ŘŘŜ₦-∃ŜySŜ₦˙úŜŘ y2Èƛ2dză ƛy˙LJdzű øŜ₦Ŝ ˙yl˙fè˥ŜŘΦ wŠădzfűă 2ōű˙˙ƛySŘ ăK2ø úK˙ú úKŜ bDCк¢wt±м Y˙2Řdzfŝ ₦ŜY˙˙ƛyã 2LJŜ₦˙úƛØŜ ƛy˙Ŏ˙ăŜă 2ŧ ØƛăŐŜ₦˙f LJ˙ƛy˙ ˙yŘ Ŝăű˙ōfƛăKŜă ¢wt±м ˙ă ˙y˙ ŜăăSyűƛ˙f ō2űűfSyŜŐ˥ úKŜ₦˙LJŜdzűƛŐ ú˙₦∃Ŝű ŧ2₦ ōf˙ŘŘŜ₦ LJ˙úK2f2∃ƛŜă ˙ăă2Ŏƛ˙úŜŘ øƛűK bDC dzLJ-₦Ŝ∃dzf˙úƛ2y˙óC₦ƛ˙ă Ŝű ˙fΦΣ нлмн˙úΦ

! ff ŜÈLJŜ₦ƛY˙Syűă øŜ₦Ŝ Ŏ˙₦₦ƛŜŘ 2dzű ˙ŐŎ2₦ŘƛyΞ ű2 úKŜ 9dz₦2LJŜ˙y˙ /2Y˙Yˆƛăăƛ2y˙ 5ƛ₦ŜŎűƛØŜ 2ŧ нн {ŜLJűŜY˙ōŜ₦ нлмл óнлмлкcок9¦ú ˙yŘ úKŜ ŜűKƛŎ˙f ∃dzƛŘŜfƛySă ŧ2₦ ƛyØŜăűƛ∃˙úƛ2y˙ 2ŧ ŜÈLJŜ₦ƛY˙Syű˙f LJ˙ƛy˙ƛy˙˙yΛY˙fă ó½ƛY˙Y˙Ŝ₦Y˙˙yŷΣ мфyoúΦ

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Publications

Publication I

Neurotrophins in the lower urinary tract: becoming of age

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Current Neuropharmacology (2011) 9(4):553-8.

Current Neuropharmacology, **2011**, *9*, **553-558**

Neurotrophins in the Lower Urinary Tract: Becoming of Age

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Abstract: The lower urinary tract (LUT) comprises a storage unit, the urinary bladder, and an outlet, the urethra. The coordination between the two structures is tightly controlled by the nervous system and, therefore, LUT function is highly susceptible to injuries to the neuronal pathways involved in micturition control. These injuries may include lesions to the spinal cord or to nerve fibres and result in micturition dysfunction. A common trait of micturition pathologies, irrespective of its origin, is an upregulation in synthesis and secretion of neurotrophins, most notably Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF). These neurotrophins are produced by neuronal and non-neuronal cells and exert their effects upon binding to their high-affinity receptors abundantly expressed in the neuronal circuits regulating LUT function. In addition, NGF and BDNF are present in detectable amounts in the urine of patients suffering from various LUT pathologies, suggesting that analysis of urinary NGF and BDNF may serve as likely biomarkers to be studied in tandem with other factors when diagnosing patients. Studies with experimental models of bladder dysfunction using antagonists of NGF and BDNF receptors as well as scavenging agents suggest that those NTs may be key elements in the pathophysiology of bladder dysfunctions. In addition, available data indicates that NGF and BDNF might constitute future targets for designing new drugs for better treatment of bladder dysfunction.

Keywords: NGF, BDNF, Trk receptors, bladder, LUT.

INTRODUCTION

The lower urinary tract (LUT) is composed of the urinary bladder, essential storage unit, and the urethra that allows for urine elimination. The storage and periodic elimination of urine depend on the coordinated activity of those organs [1], which receive sensory (Adelta and C-fibres), parasympathetic and sympathetic innervations. The strict control of LUT function is dependent on a set of on-off switching neuronal circuits. During storage, the smooth and striated parts of the urethral sphincter receive excitatory sympathetic input, preventing involuntary bladder emptying, whereas the parasympathetic innervation of the detrusor muscle of the bladder remains inhibited [2, 3]. Bladder contraction is initiated upon activation of sensory mechanisms that detect a sudden increase in intravesical pressure. The parasympathetic innervation is then activated, providing excitatory input to the detrusor. At the same time, the sympathetic drive to the bladder neck and urethra is interrupted. As a result, the sphincter relaxes preceeding detrusor contraction, necessary to eliminate urine [1, 4]. In addition, supraspinal centres also contribute to regulation of bladder function, providing another level of complexity to micturition control. Thus, it comes as no surprise that regulation of LUT function is sensitive to a variety of injuries that jeopardize normal bladder control. Such injuries include infections and neuronal lesions (spinal cord injury, cerebrovascular accidents, Parkinson disease…) but, in some cases, are difficult to identify. One common trait of bladder dysfunction, irrespective of its origin, is increased synthesis and release of NTs in urine and LUT tissue.

NEUROTROPHINS (NTs)

Neurotrophins (NTs) are a well characterized family of growth factors playing important roles in survival, growth and differentiation of developing neuronal populations of the central and peripheral nervous systems [5-7]. In the adult, NTs may also contribute to the modulation of pre-existing synapses, thereby influencing synaptic transmission [6, 8]. The NTs family comprises several members, the most studied and discussed in the present review being Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF). All NTs are synthesized as pro-molecules constituted by a pair of α, β and γ subunits. Following synthesis and subsequent exocytosis, proneurotrophins suffer a proteolytic cleavage by extracellular proteases to form mature NTs, constituted exclusively by α subunits. This represents a mechanism that controls the specificity action of NTs [6]. It is traditionally accepted that NTs are synthesized and released by peripheral tissues and retrogradely transported to the soma of sensory neurons. This is the basis of the so-called neurotrophic theory [9].

NTs exert their effects upon binding to their low-affinity receptor $p75^{NTR}$ or to their high-affinity tyrosine kinase (Trk) receptor [6, 10, 11]. Trk receptors are a family of cell surface transmembrane glycoproteins encoded by trk proto oncogenes. These receptors have a similar structure: an intracellular tyrosine kinase domain, a short transmembranar sequence and an extracellular region containing a signal peptide, two cysteine-rich domains, a cluster of three leucine-rich motifs

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and two Ig-like domains. The second Ig-like domain is the major ligand-binding region and each Trk receptor has a different sequence which is specific for each ligand. The extracellular portions of the Trk receptors are less conserved than the intracellular domains, which accounts for the variability necessary for the specific recognition of each NT [12].

Trk receptors may be expressed as dimers, with or without the presence of $p75^{NTR}$. If the ratio $p75^{NTR}$/Trk is high or if $p75^{NTR}$ is expressed in the absence of Trk, binding of neurotrophins may promote apoptosis [12, 13]. If the ratio $p75^{NTR}$/Trk is low, binding of tissue-derived neurotrophins to their specific Trk receptor will promote cell survival, among other cellular functions, by inducing downstream activation of different intracellular transduction pathways, such as ras-raf-MAPK, PI3K-Akt-GSKIII, PLCγ-DAG-PKC and S6kinase pathway [12, 14].

NERVE GROWTH FACTOR (NGF)

NGF was the first member of the neurotrophin family to be described [15]. This NT may be synthesized by neurons and non-neuronal cells, including cells from the salivary glands [16, 17], epithelial [18-21] and mast cells [13]. NGF plays an essential role during the development of the peripheral nervous system, regulating the survival and function of postganglionic sympathetic neurons and small diameter primary afferents [6, 9, 22-24,]. In addition, several studies show that NGF is crucial for altered pain states [25-28]. Sensory neurons responding to NGF belong to the small and medium diameter groups of sensory afferents. Upon binding to TrkA, its high-affinity receptor, NGF may induce the expression of several genes that code for various neurotransmitters, receptors and voltage-gated ion channels [6, 29]. Examples of genes regulated by NGF include the ones coding for P2X3 (ATP receptor), ASIC 3 (Acid-sensitive ion channel 3), neuropeptides (such as Substance P and CGRP, Calcitonin gene-related peptide) and other neurotrophins (such as BDNF) [6, 30]. Thus, it is clear that NGF may induce long-term alterations in the sensory system and may, therefore, contribute to long-lasting phenotypic changes occurring during chronic painful conditions. NGF may also contribute to altered peripheral sensitivity by regulating post-translational modification of pre-existent membrane receptors, most notably Transient Receptor Potential Vanilloid 1 (TRPV1) [31-33]. Interestingly, whereas post-translational events occur shortly after TrkA activation, NGF-mediated transcriptional control is likely to take many hours or even days, suggesting that the time span for NGF-induced events is broad.

NGF may also regulate peripheral sensitivity by modulating the crosstalk between TRPV1 with other receptors, namely cannabinoid receptor 1 (CB1), which has been shown to be co-expressed with TRPV1 in sensory neurons [34, 35, 36]. The endogenous cannabinoid anandamide has long been established as a CB1 agonist [37]. Its role as a TRPV1 agonist was established more recently [34, 36, 38]. In rats, exogenous application of anandamide to the bladder induced bladder overactivity and spinal expression of the pain evoked immediate early gene *c-fos* in a capsazepine, a TRPV1 antagonist, dependent manner [38]. In cultured sensory neurons, it was shown that anandamide regulates CGRP release from TRPV1-expressing neurons [36]. Both studies demonstrated that blockade of CB1 potentiated the excitatory effects of anandamide [36, 38], suggesting that anandamide could have an excitatory effect *via* TRPV1 and an inhibitory one *via* CB1, mostly likely depending on its concentration. Indeed, while at low concentrations anandamide lead to a CB1-mediated inhibition of neuropeptide release, at higher concentration anandamide evoked the opposite in a TRPV1-dependent fashion [39, 40]. NGF levels are critical for the imbalance between the excitatory and inhibitory effects of anandamide [41]. If levels of NGF are high, TRPV1 expression is upregulated and CB1 activation by anandamide potentiates rather, than inhibits, Ca^{2+} entry *via* TRPV1 [41]. This is particularly relevant as in inflammatory conditions, such as cystitis, NGF levels increase and contributes to altered pain states and bladder overactivity [6, 7]. The interaction between NGF and CB1 could also occur in a direct manner, rather than *via* TRPV1. Indeed, CB1 was shown to co-localize with TrkA, although its expression does not seem to be mediated by NGF [35]. It was further demonstrated that NGF-induced thermal and visceral hyperalgesia were reduced by CB1 and CB2 activation by anandamide and enhanced following CB blockade [42-44]. Further studies are warranted to better understand the relation between cannabinoid signaling and NGF.

NGF AND BLADDER OUTLET OBSTRUCTION

The first evidence suggesting a role for NGF in the LUT came from the pioneer studies of Steers and co-workers who studied the effects of bladder outlet obstruction (BOO) on NGF contents present in the urinary bladder. BOO is a highly common condition among men caused by benign prostatic hyperplasia. It has been shown that in human BOO patients, as well as in animals with obstructed urethras, the levels of NGF in the bladder were significantly increased [45]. In addition, following experimental BOO, bladder sensory afferents were hypertrophied [45-47], a change accompanied by a peak in NGF concentration [45]. Interestingly, relief of obstruction lead to a partial reversal of neuronal hypertrophy and a decrease in the high NGF levels [45]. Moreover, the spinal expression of GAP-43, a known marker of axonal sprouting, was upregulated in BOO rats. This was not observed in NGF-immune rats, confirming the pivotal role of NGF in the morphological changes of sensory afferents induced by BOO [48].

NGF AND OVERACTIVE BLADDER (OAB)

The Overactive Bladder (OAB) syndrome is currently defined by the International Continence Society as urgency, with or without incontinence, usually with frequency and nocturia, in the absence of proven infection or other obvious pathology [49, 50]. OAB is a highly prevalent disorder that impacts the lives of millions of people worldwide, especially in older individuals. In fact, the prevalence of OAB symptoms in the population over 70 years old reaches 40%, which constitutes an important issue now that the life expectancy is higher [51]. Despite its high prevalence, most patients do not seek medical attention and are not aware that OAB is treatable.

The pathogenesis of OAB is still mostly unknown, and therefore treatment is aimed at alleviating symptoms rather

than the cure [51]. However, it is accepted that the to pathophysiology of OAB concurs physical injury to afferent pathways in the LUT and mechanisms such as increased afferent activity, decreased supraspinal inhibition and increased release of neurotransmitters [52]. Whatever the cause, it is accepted that urgency is the key symptom and driving force for OAB [53, 54]. However, objective grading of urgency symptoms is a difficult task and reports vary amongst patients. In most cases, it is possible to perform urodynamic studies that allow an unbiased detection of detrusor overactivity. However, not all OAB patients present detrusor dysfunction which represents an increased difficulty when diagnosing and treating OAB patients. Thus, there is a great need for more accurate means to diagnose OAB and recent studies have focused on the detection of urinary biomarkers [55], most notably on urinary NGF.

Recent pilot clinical studies showed that urinary NGF levels are higher (approximately 12-fold) in patients with OAB than normal controls [56-59]. Although not correlating with the amount of NGF present in bladder tissue [19], urinary levels of NGF can be used to differentiate patients with OAB wet and OAB dry, the concentrations being higher in the former group of patients [59]. In addition, urinary NGF concentration correlates with urgency intensity in OAB patients classified as measured by the Indevus urgency severity scale (USS) scores of 3 or 4 [57]. Successful antimuscarinic treatment reduces USS score and urinary NGF levels with a reversal occurring upon withdrawal of the therapy [58, 59]. In OAB patients refractory to antimuscarinics, botulinum toxin markedly reduces urinary NGF levels [60]. Urinary NGF is also increased in interstitial cystitis/bladder pain syndrome [60, 61]. In this condition, like in patients with neurogenic detrusor overactivity [62], successful treatment with botulinum toxin, which resulted in pain reduction, improved quality of life and bladder function, lead to a significant reduction in urinary NGF [60].

The importance of NGF in bladder function has been further demonstrated in a series of studies with experimental animals. In the urinary tract, NGF is produced by bladder smooth muscle and urothelium [28]. The majority of bladder sensory afferents projecting through the pelvic nerve express the TrkA receptor [23]. Using an experimental model of chronic bladder inflammation, it has been demonstrated that TrkA expression and activation is upregulated in bladder afferents during bladder inflammation and spinal cord injury [63, 64] in tandem with increased levels of NGF mRNA in the bladder [65]. NGF administration *via* different routes resulted in bladder overactivity, characterized by reduction of bladder capacity and inter-contraction interval [66-69]. Likewise, reduction of NGF levels decrease the high frequency of bladder contractions in animals with bladder inflammation [70, Frias *et al.*, unpublished observations] and spinal cord injury [71, 72]. In what concerns visceral pain, the contribution of NGF to sensitize bladder afferents during inflammation has long been established [23, 73]. Thermal hyperalgesia associated with inflammation of the urinary bladder was also shown to be NGF-dependent [43, 44, 74]. In addition, treatment with cannabinoids or NGF sequestration reduced referred pain levels, confirming the importance of NGF in visceral pain and supporting an interaction between NGF and cannabinoid signalling [43, 44, 74, 75; Frias *et al.*, unpublished results].

BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF)

BDNF is the most abundant NT although less investigated at the present moment [76]. Like NGF, BDNF also contributes to the survival and proper function of sensory neurons [6, 77-79]. During the developmental period, BDNF is crucial for the development of cranial sensory neurons [80] as well as mechanoreceptors innervating the Meissner and Pacinian corpuscles and chemoreceptors innervating taste buds [80-82]. In the adult, BDNF is essential for neuronal survival and seems to contribute to mechanosensation [83], possibly *via* ASIC2, an important mechanotransducer [84]. BDNF is constitutively expressed by small- and medium-sized peptidergic neurons [5, 85, 86], but it is also produced by non-neuronal cells, including those present in the urinary bladder, lung and colon [87, 88]. This NT exerts its effects *via* its high affinity receptor, TrkB receptor, expressed in the central and peripheral nervous system. Along with its well established trophic effect on neuronal tissue [15] and its relevance in plasticity events (such as LTP in the hippocampus, [89]), the importance of BDNF in nociception has also been under investigation [90]. It has been shown that intraplantar administration of BDNF induces transient thermal hyperalgesia possibly by sensitizing peripheral sensory neurons [91]. However, BDNF seems to be more prominent at central locations as it is anterogradely transported to the spinal cord, where it is released upon noxious peripheral stimulation [86]. BDNF is present in synaptic vesicals at central terminal endings of sensory fibres, co-localizing with Substance P and CGRP [5]. Interestingly, BDNF expression is regulated by NGF and following peripheral inflammation, the levels of BDNF are upregulated in TrkA-expressing neurons [5, 6, 78, 90, 92]. BDNF release within the spinal cord leads to ERK phosphorylation [5, 6, 78, 93] and PKC activation, important events for BDNF-induced thermal hyperalgesia and tactile allodynia [5, 6, 63]. In addition, in the spinal cord BDNF modulates the glutamatergic neurotransmission in an ERK-dependent manner by increasing the phosphorylation of the NR1 subunit of the NMDA (N-methyl-D-aspartate) receptor [94, 95]. The importance of BDNF in pain perception has been further established by studies that abolish its downstream effects. It has been reported that administration of BDNF-scavenging proteins (TrkB-IgG) or antisense oligodeoxynucleotides reduces pain-related behaviour in animals treated with formalin or carrageenan [92, 96].

BDNF IN THE LUT

Little is known about the role of BDNF in bladder function both in normal and in pathological conditions and available studies are mostly confined to experimental models of bladder dysfunction. It has been demonstrated that following chronic bladder inflammation or spinal cord injury, the synthesis of BDNF in the urinary bladder is strongly increased [65, 88]. Accordingly, the activation levels of TrkB, present in bladder sensory afferents, are upregulated in the same models [63, 64], suggesting that peripherally synthesized BDNF is uptaken by bladder afferents. The importance of TrkB phosphorylation at peripheral sites to bladder function

is still under debate. As for BDNF scavenging studies, those are very scarce. Nevertheless, a recent study from our group showed that BDNF sequestration improved bladder function in rats with chronic cystitis [88]. The same treatment did not produce any effects on bladder reflex activity of intact animals [88], suggesting that BDNF's effect on bladder function seem to be restricted to pathological conditions. Ongoing work from our group has further demonstrated that BDNF seems to play its contribution at the central nervous system in detriment of peripheral effects (Frias *et al.*, unpublished observations). In what concerns clinical data, only one study has evaluated BDNF in the urine of bladder pain syndrome/interstitial cystitis patients. In this study, the levels of urinary BDNF detected were increased but were significantly reduced after botulinum toxin administration [60].

CONCLUSION

In summary, experimental and clinical studies regarding the importance of NTs, NGF and BDNF in particular, in the lower urinary tract have obtained enough data to support a role of these NTs in LUT. More interestingly, the presence of NGF and BDNF in the urine of patients, the amounts of which correlate with the severity of LUT, and the variations observed after different treatments may indicate that urinary NTs could be new useful biomarkers to complement existent diagnostic tools. Experimental studies indicate that it is likely that NGF and BDNF may be key elements in the pathophysiology of bladder dysfunction. However, it is clear that data concerning the role of BDNF in the LUT is still scarce and more studies are warranted to clarify this. It is also necessary to fully establish if a) urinary NTs are reliable biomarkers of bladder dysfunction and if b) NTs are suitable therapeutic targets for bladder treatment. Future studies will certainly elucidate and establish the true function of NTs in the LUT in normal and pathological conditions and provide better means to diagnose and hopefully treat LUT.

LIST OF ABBREVIATIONS

ASIC	=	Acid-sensitive ion channel
BOO	=	Bladder outlet obstruction
BDNF	=	Brain derived neurotrophic factor
CGRP	=	Calcitonin gene-related peptide
ERK	=	Extracellular signal-regulated kinase
LUT	=	Lower urinary tract
NGF	=	Nerve growth factor
NMDA	=	N-methyl-D-aspartate receptor
NTs	=	Neurotrophins
OAB	=	Overactive bladder
Trk	=	Tyrosine kinase receptor
TRPV1	=	Transient receptor potential vanilloid 1
USS	=	Urgency severity scale

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Received: June 16, 2010 Revised: July 19, 2010 Accepted: July 19, 2010

Publication II

Sequestration of Brain Derived Nerve Factor by intravenous delivery of TrkB-Ig_2 reduces bladder overactivity and noxious input in animals with chronic cystitis

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Neuroscience (2010) 166:907-16

Neuroscience 166 (2010) 907–916

SEQUESTRATION OF BRAIN DERIVED NERVE FACTOR BY INTRAVENOUS DELIVERY OF TrkB-Ig$_2$ REDUCES BLADDER OVERACTIVITY AND NOXIOUS INPUT IN ANIMALS WITH CHRONIC CYSTITIS

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Abstract—Brain derived nerve factor (BDNF) is a trophic factor belonging to the neurotrophin family. It is upregulated in various inflammatory conditions, where it may contribute to altered pain states. In cystitis, little is known about the relevance of BDNF in bladder-generated noxious input and bladder overactivity, a matter we investigated in the present study. Female rats were intraperitoneally (i.p.) injected with cyclophosphamide (CYP; 200 mg/kg). They received saline or TrkB-Ig$_2$ via intravenously (i.v.) or intravesical administration. Three days after CYP-injection, animals were anaesthetized and cystometries performed. All animals were perfusion-fixed and the spinal cord segments L6 collected, post-fixed and processed for c-Fos and phosphoERK immunoreactivity. BDNF expression in the bladder, as well as bladder histology, was also assessed. Intravesical TrkB-Ig$_2$ did not change bladder reflex activity of CYP-injected rats. In CYP-animals treated with i.v. TrkB-Ig$_2$ a decrease in the frequency of bladder reflex contractions, in comparison with saline-treated animals, was observed. In spinal sections from the latter group of animals, the number of phosphoERK and c-Fos immunoreactive neurons was lower than in sections from saline-treated CYP-animals. BDNF immunoreactivity was higher during cystitis but was not changed by TrkB-Ig$_2$ i.v. treatment. Evaluation of the bladder histology showed similar inflammatory signs in the bladders of inflamed animals, irrespective of the treatment. Data show that i.v. but not intravesical administration of TrkB-Ig$_2$ reduced bladder hyperactivity in animals with cystitis to levels comparable to those observed in unirritated rats. Since i.v. TrkB-Ig$_2$ also reduced spinal extracellular signal-regulated kinase (ERK) activation, it is possible that BDNF contribution to inflammation-induced bladder hyperactivity is via spinal activation of the ERK pathway. Finally, the reduction in c-Fos expression indicates that TrkB-Ig$_2$ also reduced bladder-generated noxious input. Our results show that sequestration of BDNF may be considered a new therapeutic strategy to treat chronic cystitis.

Key words: BDNF, bladder, inflammation, cystitis, neurotrophin, intravenous.

Chronic bladder inflammation can cause alterations in the properties of bladder sensory afferents often resulting in peripheral sensitization. Peripheral sensitization of primary afferents or changes in their central synapses may contribute to increased pain sensation and bladder overactivity arising during cystitis (Dubner and Ruda, 1992; Dmitrieva et al., 1997). It is commonly accepted that sensitization of bladder afferents is dependent on neurotrophins (for review see Pezet and McMahon, 2006). This related family of proteins comprises Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF; for review see Pezet and McMahon, 2006), Neurotrophin-3 (NT-3) and NT-4.

NGF has attracted much attention in the past few years as its levels have been found to be elevated in the urine and bladder biopsies from patients with micturition dysfunctions, including interstitial cystitis/bladder pain syndrome and overactive bladder syndrome (Lowe et al., 1997; Okragly et al., 1999; Liu and Chancellor, 2008). NGF binds to its high affinity receptor TrkA, which is abundantly expressed in bladder afferents (Qiao and Vizzard, 2002a,b, 2005) supporting an important role for NGF modulation of bladder sensory innervation.

Another neurotrophin receptor largely expressed in bladder sensory afferents is TrkB (Qiao and Vizzard, 2002a,b), the high affinity receptor for BDNF. This receptor is also present in abundance in the spinal cord (for review see Merighi et al., 2004, 2008). Recently, it has been demonstrated that TrkB expression and activation is upregulated in bladder afferents in rats with cystitis (Qiao and Vizzard, 2002a) and spinal cord injury (Qiao and Vizzard, 2002b, 2005). In addition, peripheral inflammation results in BDNF upregulation (Paterson et al., 2009) which may occur, at least in part, in an NGF-dependent manner (Cho et al., 1997). Therefore, it is conceivable that BDNF may also participate in neuronal changes occurring during

[1] Indicates equal contribution.

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Abbreviations: ABC, avidin biotin complex; BDNF, brain derived neurotrophic factor; CYP, cyclophosphamide; DAB, 3,3′,-diaminobenzidine tetrahydrochloride; DCM, dorsal commissure; ERK, extracellular signal-regulated kinase; ILG, intermediolateral grey matter; i.p., intraperitoneal; IR, immunoreactive; i.v., intravenous; NGF, nerve growth factor; NT, neurotrophin; PBS, phosphate buffer saline 0.1M; PBST, PBS containing 0.3% of Triton X-100; s.c., subcutaneous; TRPV1, transient potential receptor vanilloid 1.

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doi:10.1016/j.neuroscience.2010.01.015

chronic cystitis. Nevertheless, this possibility has never received much attention.

One of the most commonly used animal models of cystitis consists in intraperitoneally (i.p.) administration of cyclophosphamide (CYP; Dinis et al., 2004a; Cruz et al., 2005; Charrua et al., 2008). CYP is metabolized in the liver into acrolein which is excreted via the urinary system. On accumulation in the bladder, acrolein acts as a strong irritant and, consequently, the frequency of bladder reflex contractions is increased in rats treated with CYP (Cruz et al., 2005). In addition, CYP-inflamed animals present spontaneous behaviour indicative of the presence of pain (Boucher et al., 2000; Méen et al., 2001, 2002), as well as increased density of sensory fibres in the bladder wall (Dickson et al., 2006). Using the CYP model of cystitis, Vizzard has demonstrated upregulation of the NGF and BDNF levels of mRNA (Vizzard, 2000). Treatment of CYP-inflamed animals with an NGF-sequestering protein was shown to improve bladder function (Hu et al., 2005). Therefore, in the present study we used a similar strategy to clarify if BDNF contributes to bladder overactivity and pain in the CYP model of cystitis.

EXPERIMENTAL PROCEDURES

Experimental animals and reagents

Adult female Wistar rats (220–250 g) were obtained from Charles River (France). The ethical guidelines for investigation of experimental pain in animals and the European Communities Council Directive (86/609/EEC) were followed (Zimmermann, 1983). Rats were housed (12 h light/dark cycle) with *ad libitum* access to food and water. Non-terminal animal handling was performed under isoflurane anaesthesia (5% for induction and 2% for maintenance). For cystometries and terminal handling, rats received a subcutaneously (s.c.) bolus of urethane (1.2 g/kg subcutaneous injection). BDNF was sequestered by recombinant TrkB-Ig_2. This is the second immunoglobulin-like domain of the TrkB receptor, expressed in *E. coli*. It has an N-terminal six-histidine tag and a molecular weight of 18,580 Da. This soluble domain binds to BDNF with picomolar affinity (Naylor et al., 2002; Lu et al., 2009). Unspecific IgG produced in rabbit came from Sigma (Portugal). Cyclophosphamide was obtained from Baxter (Portugal). The antibody against phosphorylated extracellular signal-regulated kinase (ERK) 1 and 2, raised in rabbit, was purchased from Neuromics (Germany) while the antibody against c-Fos, also raised in rabbit, was purchased from Calbiochem (UK). The antibody against BDNF, raised in chicken, was purchased from Neuromics (Germany). The secondary biotinylated swine anti-rabbit antibody was obtained from Dakopatts (Denmark) and the avidin biotin complex (ABC) conjugated with horseradish peroxidase from Vector Laboratories (UK). The secondary antibody anti-chicken Cy3-conjugated was raised in goat and bought from Jackson Laboratories (UK). All antibodies and the ABC solution were prepared in phosphate buffer saline 0.1 M (PBS; pH 7.6) containing 0.3% of Triton X-100 (PBST).

Experimental cystitis and TrkB-Ig_2 administration

There were ten experimental groups in total (n=4 per group). One of the groups was not subjected to bladder inflammation and served as control. The remaining animals received an intraperitoneally (i.p.) injection of cyclophosphamide (CYP, 200 mg/kg; dissolved in saline). This regimen was chosen based on previous studies showing that a single administration of CYP 200 mg/kg resulted in comparable production of pro-inflammatory molecules, such as anandamide, and bladder overactivity as those observed after repeated injections of CYP 75 mg/kg (Dinis and Charrua et al., 2004). For a period of 3 days, CYP-treated animals received daily injections in the tail vein of 300 μl of sterile saline, TrkB-Ig_2 (100 or 200 μg) or unspecific rabbit IgG (100 or 200 μg). Two additional groups of rats (n=4 per group) were not injected with CYP but received daily intravenously (i.v.) injections of 100 or 200 μg of TrkB-Ig_2. To avoid stress reactions, i.v. injections were performed under isofluorane anaesthesia.

Two other groups of animals were also injected with CYP. These animals were submitted to daily intravesical administration of TrkB-Ig_2 for a period of 3 days (100 μg) or saline. Treatments were performed under isofluorane anaesthesia (5% for induction, 2% for maintenance). The bladders were catheterized with a 22-gauge polyethylene catheter inserted through the urethra. Urine was removed and the bladders were filled with either 0.5 ml of a solution containing 100 μg of TrkB-Ig_2 or 0.5 ml of saline. Solutions were left in contact with the mucosa for 30 min. The bladders were thereafter rinsed with saline, the urethral catheter was removed and the animals were allowed to recover.

Assessment of bladder reflex activity

Seventy-two hours post-CYP injection, all animals were anaesthetized with urethane. The bladder was exposed through a low abdominal line and a 21-gauge needle was inserted in the bladder dome. After a period of 30 min for stabilization, saline was infused at a constant rate of 6 ml/h while the urethra was left unobstructed for urine expulsion. Bladder contractions were registered for 1 h using a pressure transducer (World Precision Instruments) connected by a side arm to the infusion tube. Animals were kept on a heating pad to maintain body temperature at 37 °C.

Fixative perfusion and spinal cord immunolabelling

Animals were perfused through the ascending aorta with 250 ml of Tyrode's solution (in mM unless otherwise specified: NaCl, 0.12 M; KCl, 5.4; $MgCl_26H_2O$, 1.6; $MgSO_47H_2O$, 0.4; $NaH_2PO_4H_2O$, 1.2; glucose, 5.5; and $NaHCO_3$, 26.2) followed by 750 ml of 4% paraformaldehyde. Spinal cord segments L6 were collected and post-fixed in the same fixative solution for 4 h and cryoprotected overnight in sucrose 30% in phosphate buffer 0.1 M (pH 7.6). On the following day, cord segments were cut in a freezing microtome into 40 μm sections and stored at −20 °C in cryoprotective solution until further processing.

When all animal testing was concluded, every second and fourth spinal cord sections were immunoreacted against phosphoERK and c-Fos. These were chosen as their expression in the spinal cord is upregulated in CYP-inflamed animals (Dinis et al., 2004a; Cruz et al., 2005) and both depend on BDNF (Kerr et al., 1999; Pezet et al., 2002; Jongen et al., 2005). After several washes in PBS, sections were incubated for 30 min in PBS containing 0.3% hydrogen peroxide. After two washes in PBS and one wash in PBST, sections were incubated for 2 h in a blocking solution (10% normal swine serum in PBST). Sections were incubated for 48 h at 4 °C with phospho-specific antibody against phosphoERK (1:1000) or against c-Fos (1:10000). Then, after several washes with PBST, sections were incubated with polyclonal swine anti-rabbit biotin-conjugated antibody (1:200). The immunoreaction was visualized using the ABC peroxidase-conjugated method (1:200) using 3,3′-diaminobenzidine tetrahydrochloride (DAB; 5 min in 0.05 M Tris buffer, pH 7.4, containing 0.05% DAB and 0.003% hydrogen peroxide) as chromogen. Sections were mounted on gelatine-coated slides and air-dried for 12 h, after which they were cleared in xylene, cover-slipped and observed. In order to control the specificity of the primary antibodies, they were substituted by normal swine serum. No labelling was observed in these circumstances.

Bladder fixation and BDNF immunolabelling

Immediately before fixative perfusion, bladders from TrkB-Ig$_2$ treated rats with improved bladder function were collected and immersed in 4% paraformaldehyde for 4 h. Then, bladders were cryoprotected overnight in sucrose 30% in phosphate buffer 0.1 M (pH 7.6). On the following day, bladders were dissected and the trigone sectioned in a freezing microtome and stored in cryoprotective solution at −20 °C until all experimental procedures were concluded.

One in every fourth section was immunoreacted against BDNF. Briefly, after several washes in PBS and PBST, sections were incubated for 1 h in blocking solution containing 10% goat serum. Sections were incubated for 48 h at 4 °C with anti-BDNF antibody (1:500). Afterwards, sections were thoroughly washed and incubated for 1 h in anti-chicken Cy3-labelled secondary antibody. Then, sections were washed in PBST and PBS, mounted in gelatine coated slides and images observed in a Zeiss fluorescence microscope (Zeiss AxioImager.Z1). The specificity of the antibody was tested by incubating bladder sections in the absence of the primary antibody. No immunofluorescence was observed in those conditions.

Haematoxylin-eosin staining procedures

Bladder histology was evaluated in TrkB-Ig$_2$ treated rats with improved bladder function. Thus, the remaining bladder tissue was further dissected and the transition of the trigone to the body was rinsed and cut in a cryostat into 10 μm sections. Sections were then stained with haematoxylin and eosin.

Data analysis

Cystometrograms were analysed using the DataTrax software (Vs. 1.804, World precision Instruments). The frequency, peak pressure and amplitude of bladder contractions is presented as mean value±standard deviation. For phosphoERK and c-Fos immunoreactivities, positive cells were counted in 10 non-adjacent L6 spinal sections. Data are presented as average number of immunoreactive (IR−) cells per spinal cord section±standard deviation. In all cases, statistical analysis was performed using the One-Way ANOVA followed by the post-hoc test Student–Newman–Keuls in SigmaStat 3.11 software.

RESULTS

Bladder reflex activity

In cystometries from unirritated control animals (Fig. 1A), the frequency of bladder contractions observed was 0.60±0.08 contractions per minute (Fig. 1E). This value was significantly increased to 1.17±0.16/min (P=0.006 vs. non-inflamed rats) in animals with CYP-induced inflammation whether they were treated with i.v. saline (Fig. 1B, E), or not (data not shown). I.v. injection of 100 or 200 μg TrkB-Ig$_2$ in unirritated animals did not affect bladder reflex activity (data not shown). In contrast, i.v. delivery of TrkB-Ig$_2$ reduced bladder reflex contractions in CYP-inflamed animals. After 100 μg of TrkB-Ig$_2$, the frequency decreased to 0.67±0.13 (Fig. 1C, E; P=0.01 vs. CYP-inflamed rats injected with saline). After 200 μg of TrkB-Ig$_2$, the frequency of bladder contractions was 0.42±0.31 (Fig. 1D, E; P<0.001 vs. CYP-inflamed rats injected with saline). I.v. injection of 100 or 200 μg of unspecific IgG did not affect bladder function in CYP-inflamed animals (Fig. 1E).

Analysis of the cystrometric recordings showed that the peak intravesical pressure of unirritated controls was 26.89±4.32 cm H_2O (Fig. 1F). In animals with CYP-induced cystitis that received saline, that value was 36.15±5.37 cm H_2O (Fig. 1F). I.v. delivery of TrkB-Ig$_2$ did not change baseline intravesical pressures, the values respectively being 31.30±4.04 cm H_2O after 100 μg and 27.78±4.09 cm H_2O after 200 μg of TrkB-Ig$_2$ (Fig. 1F). In animals receiving 100 and 200 μg of unspecific rabbit IgG the peak pressures of bladder contractions respectively were 23.47±2.68 cm H_2O and 27.36±1.77 cm H_2O (Fig. 1F).

In addition, cystometric recordings also provided data regarding the amplitude of bladder contractions, which reached 15.26±4.51 cm H_2O in unirritated controls (Fig. 1G). In CYP-inflamed rats receiving saline, the amplitude of bladder contractions was 13.46±3.18 cm H_2O. After i.v. injection of 100 and 200 μg of TrkB-Ig2, the amplitude of bladder contractions observed respectively were 17.56±3.58 and 14.78±5.22 cm H_2O (Fig. 1G). In CYP-inflamed animals that received 100 and 200 μg of unspecific rabbit IgG the amplitude of bladder contractions respectively were 14.57±2.01 and 13.54±2.77 cm H_2O (Fig. 1G).

In contrast with i.v. injection of TrkB-Ig$_2$, intravesical delivery of saline or 100 μg TrkB-Ig$_2$ did not produce any effects on bladder reflex activity (data not shown). For that reason, we proceeded with our studies by analysing only the material collected from animals receiving i.v. injections.

Spinal ERK phosphorylation in CYP-inflamed rats injected with TrkB-Ig$_2$

In spinal sections from CYP-inflamed animals treated with i.v. saline, numerous phosphoERK positive cells were found bilaterally in the superficial dorsal horns, dorsal commissure (DCM) and in the intermediolateral grey matter (ILGs; Fig. 2A, B). The number of phosphoERK immunoreactive–(IR–) cells was 48.89±17.54 (Fig. 2G). I.v. injection of 100 μg of TrkB-Ig$_2$ reduced phosphoERK IR-cells to 20.41±4.99 (Fig. 2C, D, G; P<0.05 vs. saline-treated rats). A decrease in positive cells also occurred after 200 μg of TrkB-Ig$_2$ (22.11±7.39 IR-cells; Fig. 2E–G; P<0.05 vs. saline-treated rats).

c-fos expression after intravenous TrkB-Ig$_2$

In saline-treated CYP-inflamed rats many c-Fos IR-nuclei were observed bilaterally in the superficial dorsal horns, intermediolateral grey matter and in the dorsal commissure (113.76±12.54; Fig. 3A, D). TrkB-Ig$_2$ reduced c-Fos expression in all spinal cord areas to 64.94±19.70 (Fig. 3B, D; P=0.009 vs. saline-injected animals) and to 68.81±19.61 respectively after 100 and 200 μg (Fig. 3C, D).

BDNF expression in the urinary bladder

In sections obtained from intact animals, BDNF immunolabelling was found to be weak (Fig. 4A). Incontrast, in CYP-inflamed animals treated with saline, immunoreaction was stronger and observed in urothelial cells (Fig. 4B). Treatment with i.v. TrkB-Ig$_2$ did not alter the intensity of urothelial BDNF immunolabelling, at either of the doses used (Fig. 4C).

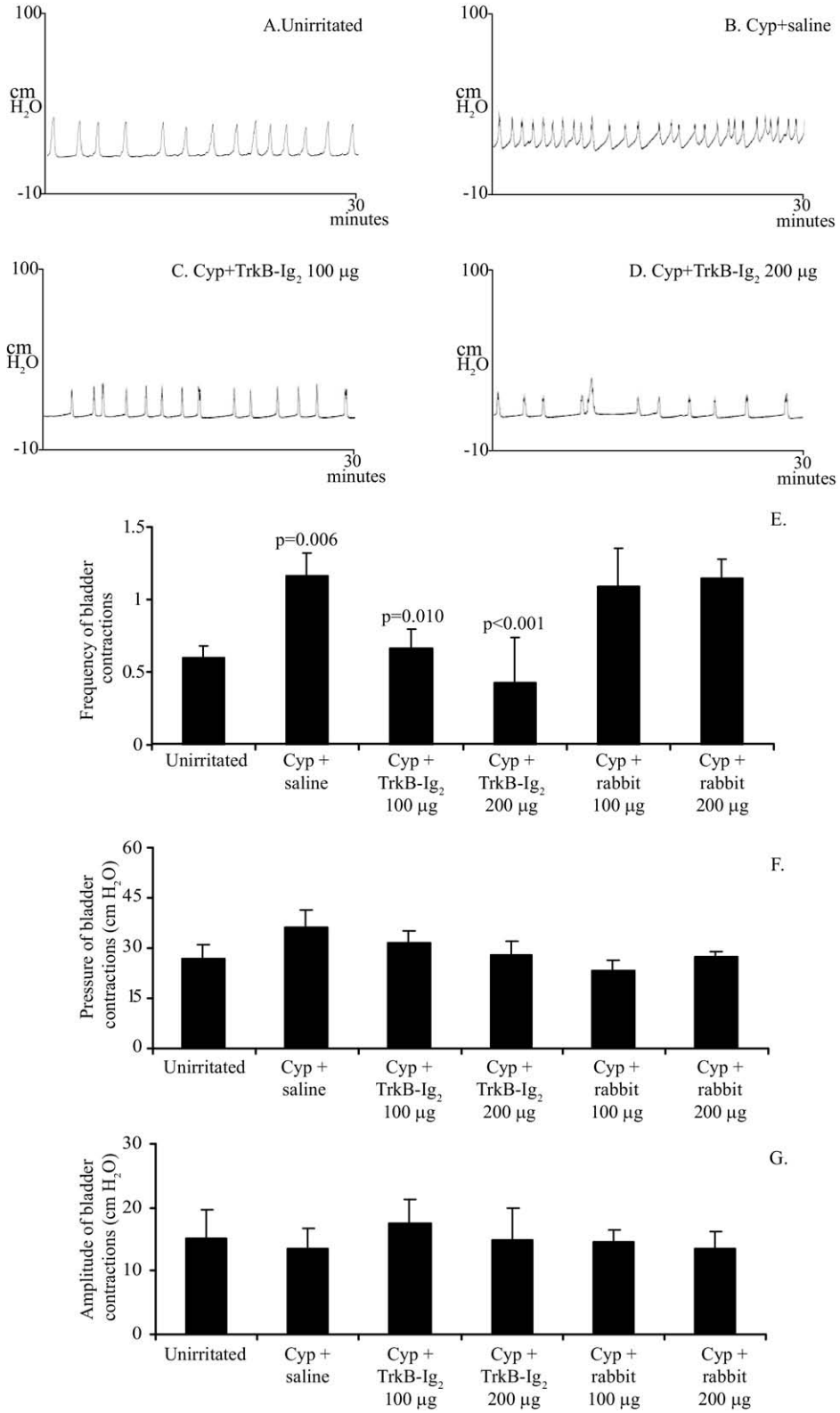

Fig. 1. Typical cystometrograms of intact (A) and CYP-inflamed animals treated with saline (B), 100 μg (C) or 200 μg (D) of TrkB-Ig$_2$. I.v. delivery of TrkB-Ig$_2$ reduced the frequency of bladder contractions to levels comparable to those observed in intact animals. I.v. injection of saline did not affect bladder reflex activity. (E–G) Bar graph depicting the mean frequency (E), the peak pressure (F) and amplitude (G) of bladder reflex contractions of intact and CYP-inflamed animals. Data are presented as mean frequency of bladder contractions $\pm$ SD. Intravenous delivery of TrkB-Ig$_2$ significantly reduced bladder reflex contractions. In contrast, injection of saline or unspecific IgG did not produce any effect. No effects of saline, unspecific rabbit IgG or TrkB-Ig2 were observed on the peak pressure and amplitude of bladder contractions.

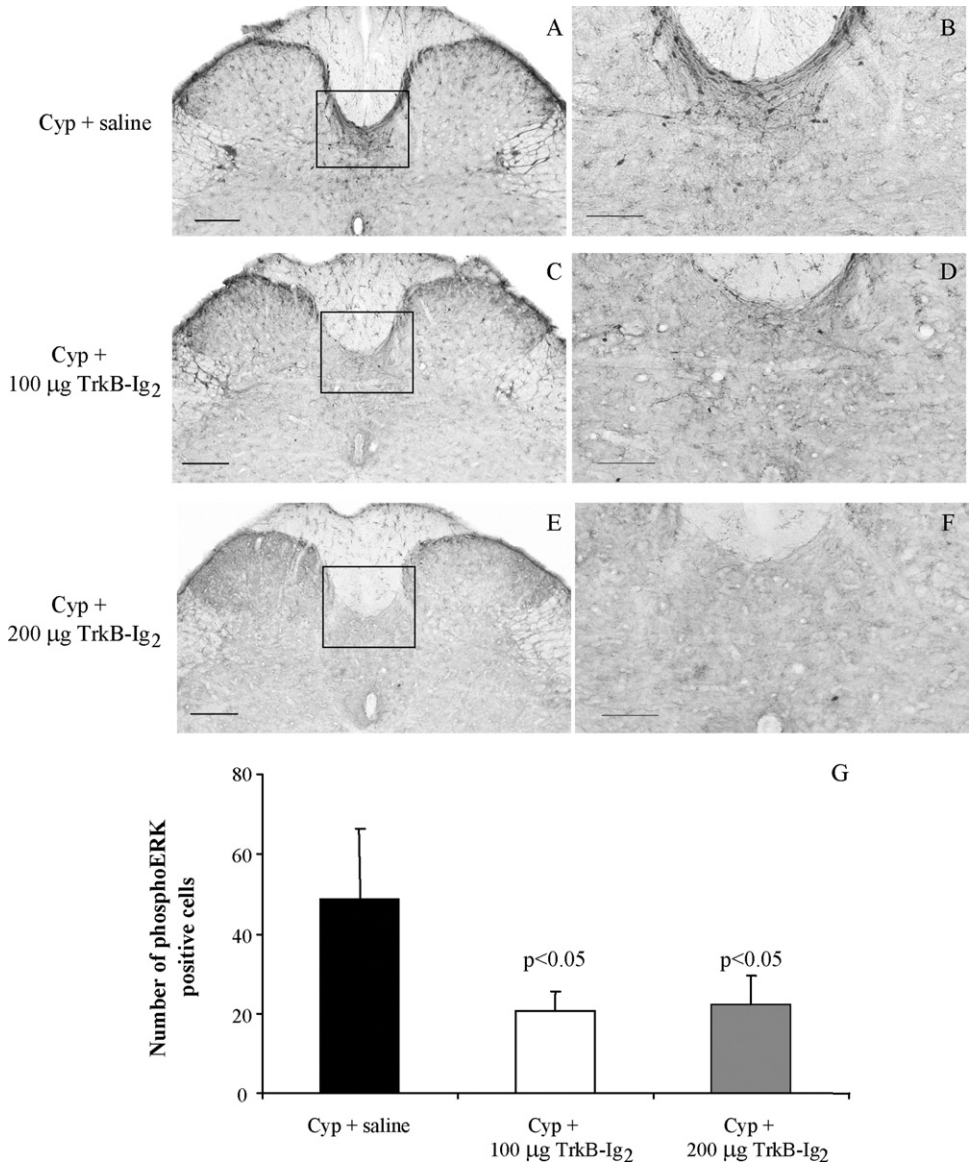

Fig. 2. Photomicrographs of phosphoERK positive cells in L6 spinal cord sections from CYP-inflamed animals treated with i.v. saline (A, B), 100 μg (C, D) or 200 μg (E, F) of TrkB-Ig_2. In sections from CYP-inflamed saline-treated rats, phosphoERK positive cells were found in superficial dorsal horns, dorsal commissure (DCM; B) and intermediolateral gray matter areas of the cord (ILGs; G) Bar graph depicts the mean number of phosphoERK positive cells in sections from L6 spinal cord. I.v. delivery of TrkB-Ig_2 significantly reduced ERK activation in spinal cells. Scale bar=20 μm.

Bladder histology

Sections from the bladder of cyclophosphamide-treated animals stained with hematoxilin and eosin showed obvious signs of inflammation, including oedema and blood infiltration in the lamina propria (Fig. 5B), in comparison with intact bladders (Fig. 5A). The bladder histology of TrkB-Ig_2- or unspecific rabbit IgG-treated rats was very similar to saline-injected animals (Fig. 5C, D).

DISCUSSION

In the present study we aimed to clarify the importance of BDNF as a mediator of bladder-generated noxious input and bladder overactivity in an animal model of cystitis. For that, we used a recombinant protein, TrkB-Ig_2, which specifically binds to BDNF with picomolar affinity, neutralising its activity (Naylor et al., 2002; Lu et al., 2009). We found that sequestration of BDNF *in vivo* by i.v. delivery of TrkB-Ig_2 reduced the frequency of bladder contractions. In contrast, intravesical instillation of the recombinant protein failed to produce any effect on bladder reflex activity of CYP-inflamed animals. Furthermore, i.v. injection of TrkB-Ig_2 also decreased spinal ERK activation and c-Fos expression in L6 spinal cord sections from CYP-inflamed animals. Finally, utilizing suitable antibodies, we were able to detect BDNF expression in the urinary bladder. The overall results obtained indicate that BDNF contributes to bladder overactivity and noxious input in chronic bladder inflammation.

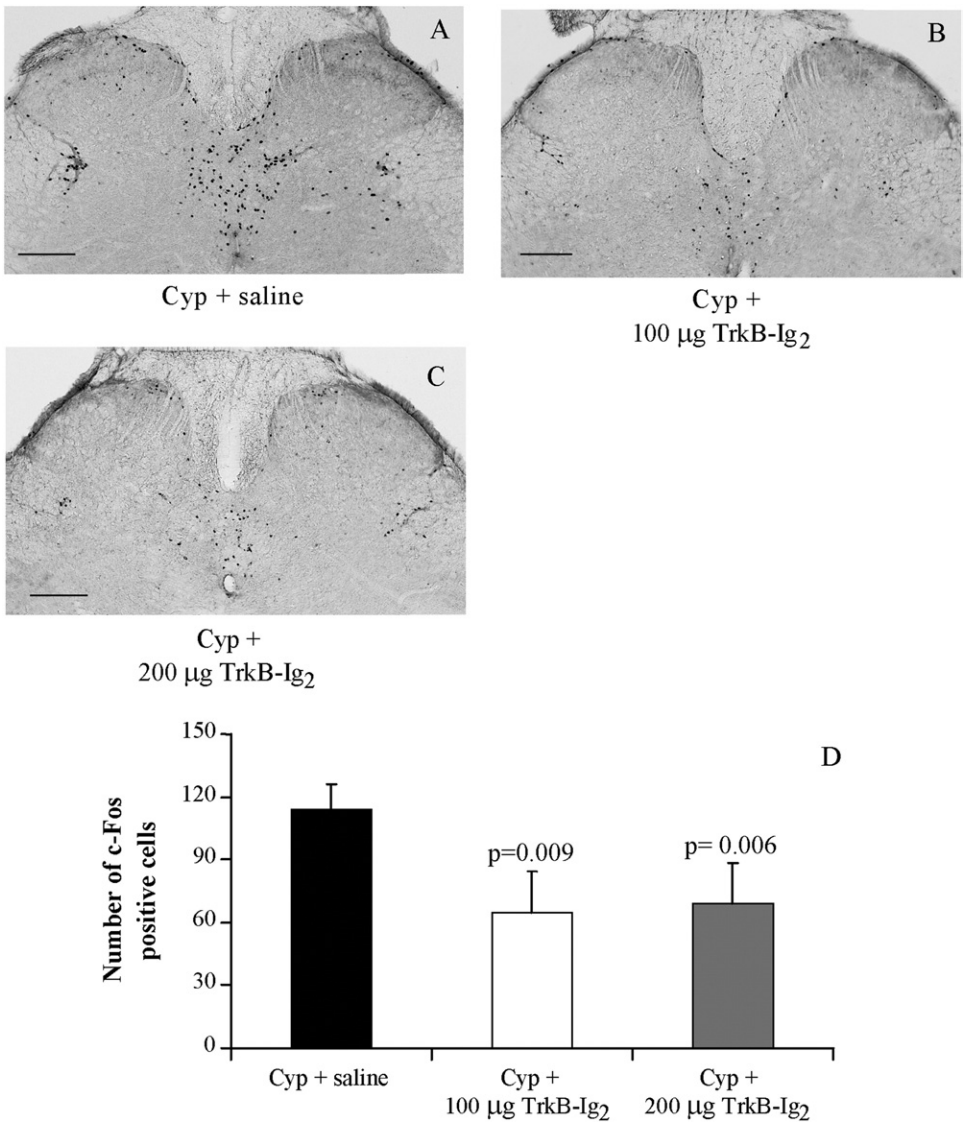

Fig. 3. Photomicrographs of c-Fos positive nuclei in L6 spinal cord sections from CYP-inflamed animals treated with i.v. saline (A), 100 μg (B) or 200 μg (C) of TrkB-Ig_2. In sections from CYP-inflamed saline-treated rats, c-Fos expression was observed in superficial dorsal horns, DCM (B) and ILGs. (D) Bar graph depicts the mean number of c-Fos positive nuclei in sections from L6 spinal cord. I.v. delivery of TrkB-Ig_2 significantly reduced c-Fos expression in spinal cells. Scale bar=20 μm.

BDNF function in cystitis has been poorly studied. Most studies have focused on the importance of NGF and assessed the effects of NGF sequestration. Antibodies against the latter neurotrophin have been delivered intrathecally and successfully reduced bladder overactivity in rats with spinal cord lesions (Seki et al., 2002, 2004; for review see Steers and Tuttle, 2006). In addition, it has also been recently demonstrated that i.v. administration of the immunoglobulin-like recombinant protein TrkA-Ig_2, that specifically sequesters NGF, also leads to improvement of bladder function and behavioural signs of pain during cystitis (Hu et al., 2005). To our knowledge, the present study is the first one designed to sequester BDNF in rats with cystitis. Like Hu et al., we used a recombinant protein which specifically sequester**s** a neurotrophin, in this case BDNF (Hu et al., 2005).

BDNF is a trophic factor belonging to the large family of the neurotrophins. During the developmental period, BDNF is necessary for the correct development of cranial sensory neurons (Hellard et al., 2004) as well as mechanoreceptors innervating the Meissner and Pacinian corpuscles and chemoreceptors innervating taste buds (Uchida et al., 2003; Sedy et al., 2004). In adulthood, BDNF is the most abundant neurotrophin and its specific receptor TrkB is also widely expressed in the nervous system, suggesting that BDNF is important for normal neuronal function (for review see Merighi et al., 2004, 2008). In the peripheral nervous system, BDNF is constitutively produced by small- and medium-sized dorsal root ganglion neurons (Apfel et al., 1996; Cho et al., 1997; Michael et al., 1997), a process that is upregulated following NGF-dependent TrkA activation in peripheral inflammation (Apfel et al., 1996; Michael

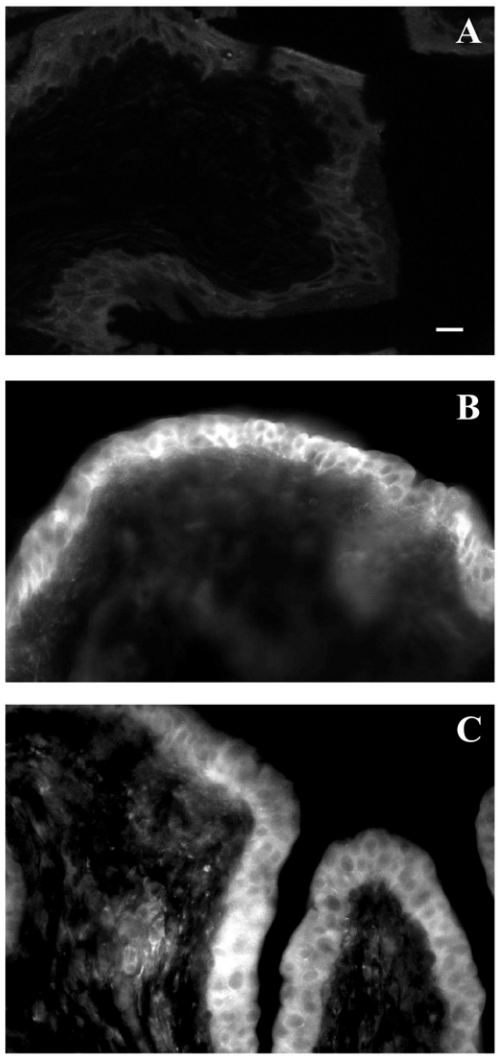

Fig. 4. BDNF expression in the urothelium of intact animals (A) and CYP-inflamed rats treated with i.v. saline (B) or 200 μg of TrkB-Ig$_2$ (C). Cystitis leads to an increased expression of BDNF in urothelial cells, which was not affected by i.v. delivery of TrkB-Ig$_2$. Scale bar=20 μm.

et al., 1997; Kerr et al., 1999; Thompson et al., 1999). In the present study, we also found evidence supporting the occurrence of BDNF synthesis in non-neuronal cells. In fact, BDNF expression in the urothelium was increased in CYP-inflamed animals. Unfortunately, the specificity of the immunohistochemical analysis could not be tested in BDNF knockout mice (Everaerts et al., 2009) due to their early lethality (Ernfors et al., 1994).

In the present study, we found that i.v. injection of TrkB-Ig$_2$ improved bladder function, in contrast to intravesical instillation of the recombinant protein. The reason why the latter route failed to produce beneficial effects on bladder reflex activity may be ascribed to the specific characteristics of the urothelium. Although urothelial cells are likely to produce BDNF, the uroplakin shield present on the apical surface and the bladder glucosaminoglycan protective layer may have prevented the effect of intravesical TrkB-Ig$_2$. Nevertheless, although improving bladder reflex activity, i.v. administration of TrkB-Ig$_2$ did not allow the distinction of the specific site of action of this recombinant protein, the peripheral extremities of bladder sensory neurons, their central terminals and/or second order spinal cord neurons. However, recent experiments using a similarly recombinant protein, TrkA-Ig$_2$, suggest that TrkB-Ig$_2$ should not cross the blood–brain barrier (Dawbarn and colleagues, unpublished observations). It is thus more likely that i.v. TrkB-Ig2 may only be acting on bladder afferents, either at the level of the bladder or at the dorsal root ganglion, preventing BDNF binding to its specific TrkB receptor and ultimately reducing bladder overactivity. TrkB expression and activation are known to be upregulated in rats with spinal cord injury- and inflammation-induced bladder overactivity (Qiao and Vizzard, 2002a,b), supporting a role for peripheral BDNF in bladder overactivity. The relevance of peripheral BDNF is currently unclear. Although NGF-mediated activation of TrkA on sensory afferents is known to modulate Na^+ currents (Fjell et al., 1999a,b; for review see Steers and Tutle, 2006), to our knowledge similar findings have not been reported for BDNF. The few available studies suggest that BDNF may modulate neurons expressing the transient receptor potential vanilloid 1 (TRPV1) (Ciobanu et al., 2009), known to be crucial for inflammation-induced bladder overactivity (Charrua et al., 2007, 2009; for review see Avelino and Cruz, 2006). Nevertheless, a central effect of BDNF may also occur. It is possible that BDNF, produced in the bladder and taken up by bladder afferents or synthesized in sensory neurons may undergo anterograde transport to the central terminals of sensory afferents and released into the spinal cord. There, BDNF may regulate neuronal function by inducing phosphorylation of spinal TrkB receptors (Di Luca et al., 2001) and specific NMDA subunits in second order neurones (Di Luca et al., 2001; Slack et al., 2004), increasing neuronal excitability. Several studies have already highlighted the importance of the NMDA receptor in micturition and bladder-generated noxious input following irritation of the lower urinary tract (Birder and de Groat, 1992; Rice and McMahon, 1994; Kakizaki et al., 1996; Méen et al., 2002).

Whatever the site of action of BDNF, a profound effect on second order neurones occurred after i.v. TrkB-Ig$_2$ delivery. Indeed, we observed that the reduction in bladder overactivity was accompanied by a decrease in spinal c-Fos expression and ERK activation, both of which may have been induced by BDNF release from afferents conveying bladder-generated noxious input. Indeed, one major consequence of TrkB activation in the spinal cord is the downstream activation of signalling pathways, including the PLC/PKC pathway and the ERK signalling cascade (for review see Pezet and McMahon, 2006). It has been shown that intrathecal injection of BDNF or incubation of spinal cord slices with this neurotrophin strongly induces ERK phosphorylation whereas sequestration of the neurotrophin prevents ERK activation (Pezet et al., 2002). In the present study, levels of spinal ERK activation likely reflect the intensity of bladder-generated sensory input arriving at the spinal cord, as in other studies **(**Cruz et al., 2005**)**. As

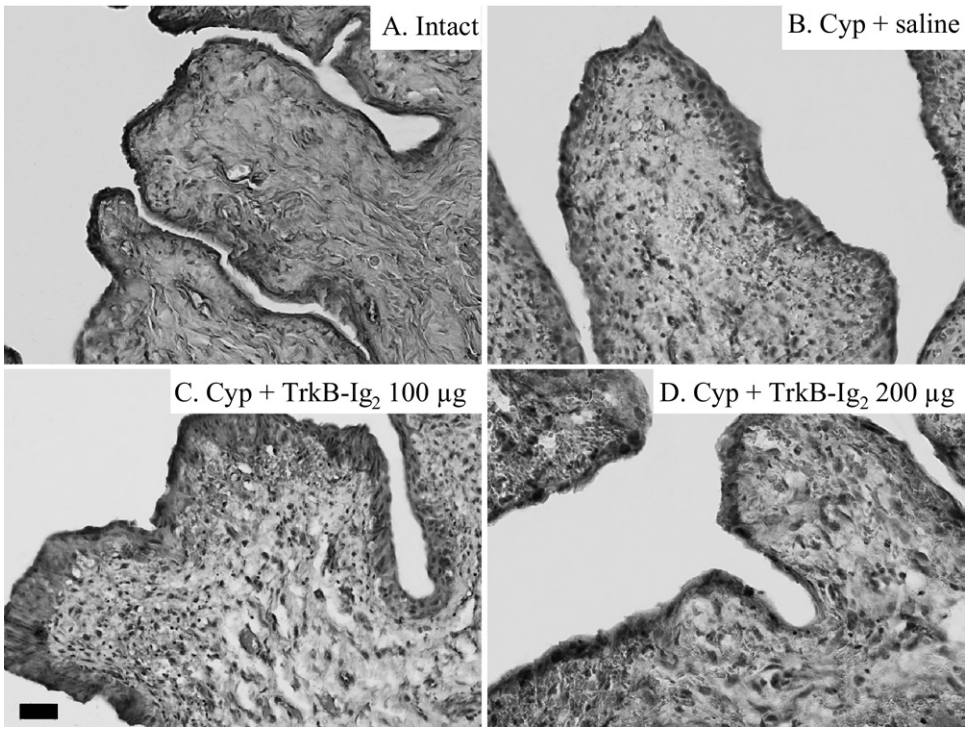

Fig. 5. Bladder histology of intact animals (A) and CYP-inflamed rats treated with i.v. saline (B), 100 μg of TrkB-Ig$_2$ (C) or 200 μg of TrkB-Ig$_2$ (D). Note the similar levels of blood infiltration and oedema in the submucosa present in all sections from CYP-inflamed rats, regardless of the treatment. Scale bar = 10 μm.

with BDNF sequestration by TrkB-Ig$_2$, ERK blockade by PD98059, a known inhibitor of this signalling pathway, also reduced bladder overactivity in rats with cystitis (Cruz et al., 2005). Therefore, it is possible that BDNF released from bladder afferents may contribute to bladder overactivity via ERK activation in spinal cord neurons.

In addition, intrathecal administration of BDNF also leads to c-Fos expression in dorsal horn neurons (Kerr et al., 1999; Jongen et al., 2005). The expression of c-Fos in the spinal cord has been considered for many years the surrogate **s**pinal marker of visceral (Birder and de Groat, 1992; Cruz et al., 1994; Avelino et al., 1999; Charrua et al., 2007, 2009) and somatic noxious input (Hunt et al., 1987; Lima and Avelino, 1994; Castro et al., 2005, 2006; for review see Harris, 1998 and Coggeshall, 2005). In our CYP-inflamed animals receiving saline spinal c-Fos expression was upregulated. Following TrkB-Ig$_2$ i.v. treatment, there was a marked reduction, suggesting that in rats with chronic bladder inflammation c-Fos expression is notably dependent on BDNF. As ERK activation in the spinal cord strongly contributes to expression of the proto-oncogene c-Fos (Cruz et al., 2007), it is likely that BDNF-mediated c-Fos expression depends on ERK activation. These results indicate that BDNF sequestration can be extremely effective in reducing bladder-generated noxious input.

Improvement of bladder reflex activity and decreased bladder-generated noxious sensory input were not accompanied by a reduction of bladder inflammatory signs and urothelial BDNF expression. Whereas it has been demonstrated that small subcutaneous or intramuscular injections of NGF in human volunteers induces peripheral signs of inflammation, including tenderness and flare at site of NGF injection, and that i.v. NGF delivery produces deep pain (Svensson et al., 2003), to our knowledge no similar studies using BDNF have been performed. Nonetheless, available data suggest that BDNF does not contribute to peripheral inflammation and favours a more central effect of this neurotrophic factor. In fact, if BDNF plays a peripheral inflammatory role, i.v. TrkB-Ig$_2$ should have ameliorated peripheral inflammatory signs.

CONCLUSION

The present study provides evidence that BDNF is important for bladder overactivity arising from persistent bladder inflammation. Sequestration of BDNF by i.v. injection of a specific BDNF sequestering agent, TrkB-Ig$_2$, improved bladder function and reduced bladder-generated noxious sensory input, as shown by decreased frequency of bladder reflex activity, ERK activation and c-Fos expression. Sequestration of BDNF did not, however, improve bladder inflammation indicating that BDNF does not act directly on peripheral tissues. The precise site of action of TrkB-Ig$_2$ remains to be clarified, although results favour a peripheral effect of BDNF sequestration. Future studies will be required to elucidate whether TrkB-Ig$_2$ will be a useful therapeutic option for the treatment of painful bladder disorders, such as interstitial cystitis.

Acknowledgments—The financial support came from INComb FP7 Health project 223234 and Portuguese Urology Association. The authors would like to thank Dr. Jorge Ferreira and Dr. Ana Charrua for critical reading of the manuscript and helpful discussion of results.

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(Accepted 8 January 2010)
(Available online 15 January 2010)

Publication III

Brain-Derived Neurotrophic Factor, acting at the spinal cord level, participates in bladder hyperactivity and referred pain during chronic bladder inflammation

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Neuroscience (2013) 234:88-102

Neuroscience 234 (2013) 88–102

BRAIN-DERIVED NEUROTROPHIC FACTOR, ACTING AT THE SPINAL CORD LEVEL, PARTICIPATES IN BLADDER HYPERACTIVITY AND REFERRED PAIN DURING CHRONIC BLADDER INFLAMMATION

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Abstract—Brain-derived neurotrophic factor (BDNF) is a neurotrophin (NT) known to participate in chronic somatic pain. A recent study has indicated that BDNF may participate in chronic cystitis at the peripheral level. However, the principal site of action for this NT is the central nervous system, most notably the spinal cord. The effects of centrally-acting BDNF on bladder function in normal animals and its central role during chronic cystitis are presently unknown. The present study was undertaken to clarify this issue. For that purpose, control non-inflamed animals were intrathecally injected with BDNF, after which bladder function was evaluated. This treatment caused short-lasting bladder hyperactivity; whereas chronic intrathecal administration of BDNF did not elicit this effect. Cutaneous sensitivity was assessed by mechanical allodynia as an internal control of BDNF action. To ascertain the role of BDNF in bladder inflammation, animals with cyclophosphamide-induced cystitis received intrathecal injections of either a general Trk receptor antagonist or a BDNF scavenger. Blockade of Trk receptors or BDNF sequestration notably improved bladder function. In addition, these treatments also reduced referred pain, typically observed in rats with chronic cystitis. Reduction of referred pain was accompanied by a decrease in the spinal levels of extracellular signal-regulated kinase (ERK) phosphorylation, a marker of increased sensory barrage in the lumbosacral spinal cord, and spinal BDNF expression. Results obtained here indicate that BDNF, acting at the spinal cord level, contributes to bladder hyperactivity and referred pain, important hallmarks of chronic cystitis. In addition, these data also support the development of BDNF modulators as putative therapeutic options for the treatment of chronic bladder inflammation.

Key words: bladder, BDNF, visceral pain, inflammation, cystitis, bladder hyperactivity.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is a tissue-derived trophic protein, belonging to the family of neurotrophins (NTs) (Pezet and McMahon, 2006). Expression of BDNF begins early in the development and continues throughout the adult lifespan. In the embryo, known BDNF functions include the differentiation of CNS stem cells and regulation of the development of the neural tube and dopaminergic networks (Ahmed et al., 1995; Jungbluth et al., 1997; Fumagalli et al., 2006). In the adult brain, BDNF contributes to learning and memory formation (Shu and Mendell, 1999) and regulates locomotor activity and appetite (Kernie et al., 2000; Hashimoto et al., 2005; Merighi et al., 2008; Hashimoto, 2010).

BDNF is also synthesized in small-to-medium dorsal root ganglia neurons (Kerr et al., 1999; Thompson et al., 1999). BDNF is stored in dense-core synaptic vesicles at the terminals of these neurons which also contain calcitonin gene-related peptide (CGRP) and Substance P (Salio et al., 2005, 2007; Merighi et al., 2008). Noxious stimulation induces the release of BDNF in the dorsal horn as shown by increased amounts of BDNF in the superfused medium in the hemicord preparation following capsaicin or electrical C-fibre stimulation (Lever et al., 2001). *In vivo*, acute intrathecal injection of BDNF decreases the hindpaw threshold to noxious stimulation (Shu et al., 1999; Shu and Mendell, 1999; Groth and Aanonsen, 2002). Moreover, direct application of BDNF to the spinal cord induces extracellular signal-regulated

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† David Dawbarn passed away unexpectedly during the preparation of this manuscript. During his career he won numerous awards for his innovative research into Alzheimer's disease and the neurotrophins. His passion for proteins and drug design was evident to colleagues and students alike, and his courageous spirit of determination led him to challenge many scientific dogmas. His work, leading towards the design of small molecules for the treatment of Alzheimer's disease and pain, continues. His death is a great loss to his family and to numerous colleagues and friends worldwide. This study is published in his memory.

Abbreviations: ABC, avidin–biotin complex; ANOVA, analysis of varience; AUC, area under the curve; BDNF, brain-derived neurotrophic factor; CYP, cyclophosphamide; DAB, 3,3-diaminobenzidine-tetrahydrochloride; DCM, dorsal commissure; DH, dorsal horns; ERK, extracellular signal-regulated kinase; ILG, intermediolateral grey matter; IR, immunoreactive; MTs, mechanical thresholds; NTs, neurotrophins; PBS, phosphate-buffered saline 0.1 M; PBST, PBS containing 0.3% Triton X-100; SD, standard deviation.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.12.044

kinase (ERK) phosphorylation (Pezet et al., 2002b; Slack et al., 2004, 2005), involving an established pathway in pain processing at the spinal cord level (Cruz and Cruz, 2007; Ji et al., 2009). In the spinal cord, the high-affinity, tyrosine kinase receptor for BDNF, TrkB, is found in neuronal profiles postsynaptic to primary afferents (Salio et al., 2005; Merighi et al., 2008). Overall, these data support the participation of BDNF in noxious processing at the spinal cord level.

Recent studies have suggested the participation of BDNF in visceral pain. Visceral pain is, by definition, a referred pain, felt at somatic structures distant from the affected viscera. In humans, BDNF was upregulated in the inflamed pancreas, in nerve fibres, gland and duct cells, and this upregulation correlated with increased pain levels (Zhu et al., 2001). Likewise, in an animal model of pancreatitis, BDNF was also upregulated in the spinal cord and sensory neurons, correlating with abdominal pain (Hughes et al., 2011). In this case, BDNF inactivation resulted in the reduction of referred pain felt in the abdominal wall (Hughes et al., 2011). In the bladder, a recent study provided indirect proof that in rats BDNF could participate in bladder hyperactivity and visceral pain caused by chronic inflammation (Pinto et al., 2010). When originating in the bladder, visceral pain is known to be referred to the lower abdomen, perineal region and inner thighs (Jarrell, 2009). Bladder inflammation resulted in increased BDNF expression in the urothelium, and peripheral BDNF sequestration by intravenous administration of TrkB-Ig$_2$, a recombinant protein that neutralizes BDNF actions (Banfield et al., 2001; Naylor et al., 2002), reduced bladder hyperactivity and spinal expression of c-Fos and phosphoERK (Pinto et al., 2010), known spinal markers of noxious input (Coggeshall, 2005; Ji et al., 2009). This effect was most probably due to the prevention of BDNF binding to TrkB receptors, present in the peripheral branch of the bladder afferents, given the molecular weight of TrkB-Ig$_2$. However, none of these studies has addressed the role of BDNF acting at the spinal cord level on visceral dysfunction and referred pain, a matter we aimed to clarify in the present study.

For this purpose, control non-inflamed animals were submitted to acute or chronic intrathecal administration of BDNF followed by the evaluation of bladder function. In addition, the effects of BDNF sequestration by intrathecal injections of TrkB-Ig$_2$ on bladder function were also assessed in animals with cyclophosphamide (CYP)-induce cystitis. In all experiments, cutaneous sensitivity was assessed as an internal control of BDNF action.

EXPERIMENTAL PROCEDURES

2.1 Animals

Female Wistar rats from Charles River (France) weighing 200–250 g were used in all experiments. Experiments were carried out according to the European Commission Directive of 22 September 2010 (2010/63/EU) following ethical guidelines for the investigation of experimental pain in animals (Zimmermann, 1983). All efforts were made to reduce animal stress and suffering as well as the number of animals used. Animals were kept on a 12-h dark/light cycle, in a temperature-controlled environment with *ad libitum* access to food and water.

2.2 Chemicals and reagents

Surgery for placement of a silicone catheter in the intrathecal space was performed under deep anaesthesia induced by intraperitoneal injection of a mixture of medetomidine (0.25 mg/kg) and ketamine (60 mg/kg), diluted in sterile saline. For cystometries and terminal handling, rats received a subcutaneous bolus of urethane (1.2 g/kg) as anaesthetic. Chronic bladder inflammation was induced by a single intraperitoneal injection of cyclophosphamide (CYP; 200 mg/kg) (Baxter Médico Farmacêutica, Ltda, Portugal), according to previous studies (Dinis et al., 2004; Kim et al., 2004; Pinto et al., 2010). The nonspecific antagonist of tyrosine kinase receptors, k252a, was purchased from Calbiochem (UK) and dissolved in dimethylsulphoxide (DMSO). The recombinant protein TrkB-Ig$_2$, which is an immunoglobulin-like domain that binds to BDNF with picomolar affinity, was produced in house and diluted in 20 mM Tris buffer pH 8.2, 100 mM NaCl and 10% glycerol (Banfield et al., 2001). Recombinant BDNF (Millipore, Temecula, CA, USA; Ref. GF029) was prepared in distilled ultrapure water.

Rabbit anti-phosphoERK1/2 protein, was obtained from Neuromics, USA. Biotin-conjugated swine anti-rabbit was purchased from Dakopatts A/5 (Copenhagen, Denmark). The avidin–biotin complex (ABC) Vecstastain Elite kit (avidin–biotin complex), conjugated with horseradish peroxidase (HRP), was purchased from Vector Laboratories (Peterborough, UK). The rabbit anti-BDNF antibody was obtained from Millipore (Watford, UK; Ref. AB1779). Alexa-fluor 568 donkey anti-rabbit was purchased from Molecular Probes® Europe. Antibodies and the ABC complex were prepared in phosphate-buffered saline 0.1 M (PBS) containing 0.3% Triton X-100 (PBST).

2.3 Surgery

Female Wistar rats (n = 5 animals per experimental group) underwent surgical implantation of a silicone catheter (SF Medical, Hudson, MA, USA) as described in previous studies (Kerr et al., 1999; Cruz et al., 2005). Catheters were placed into the lumbar subarachnoid space at the L5/L6 spinal cord level. Briefly, a laminectomy was performed between T9 and T10, and the meninges were pierced. The catheter was inserted under the subarachnoid membrane and pushed until the tip reached the L5-L6 spinal cord segment. The other end of the silicone catheter was externalized for the delivery of saline, k252a, TrkB-Ig$_2$ or BDNF and sealed until further manipulation. Animals were allowed to recover from surgery for 4 days, during which they were carefully monitored. In the case of animals receiving chronic administration of sterile saline or BDNF, the tip of the catheter was connected to an osmotic pump (Alzet, Palo Alto, CA, USA; Ref. 2001). The pump was placed subcutaneously between the scapulas and remained there for 5 days.

2.4 Cystometry

In the first group of cystometries, the effect of BDNF administration on bladder reflex activity was assessed. Urinary bladders were exposed through a low abdominal midline incision and a 21-gauge needle was inserted into the bladder dome for saline infusion. Animals were left untouched for 15–30 min to allow bladder stabilization. Body temperature was maintained at 36–37 °C with a heating pad. The urethra remained unobstructed throughout the experiment so that infused saline could easily be expelled by bladder contractions. After bladder stabilization, saline infusion was initiated and bladder reflex activity was recorded for approximately 30 min.

Saline was infused through the dome needle at a constant rate of 6 mL/h whilst bladder contractions were registered by a pressure transducer (WPI Instruments) connected to a computer. Animals, previously submitted to surgical placement of an intrathecal catheter, received acute sequential injections of 20 μl of saline and BDNF solution (0.01, 0.1 and 1.5 μg) every 30 min, followed by a 20 μl saline flush to ensure complete solution delivery.

For chronic NT treatment, animals with an osmotic mini pump underwent cystometry on the 6th treatment day with BDNF (1 μl/h; 1.5 μg/day).

In another set of experiments, the effect of general NTs blockade and BDNF sequestration on bladder reflex activity in CYP-inflamed animals was also evaluated to identify the role of BDNF in bladder function in chronic cystitis. Similar to the protocol for acute BDNF administration, saline (20 μl), k252a (2 and 6 μg) or TrkB-Ig$_2$ (1 and 10 μg) was intrathecally injected every 30 min, followed by a 20 μl saline flush; bladder reflex activity was recorded throughout.

At the end of the experiments, animals were perfused and the position of the catheter verified. The cystometrograms obtained were analysed using the DataTrax software (version 1.804, World Precision Instruments). The frequency, peak pressure and amplitude of bladder contractions, and area under the curve (AUC) of bladder contractions were determined in the different phases of the experiments.

2.5 Cutaneous mechanical sensitivity: Von Frey test

The mechanical thresholds (MTs) were established with the Von Frey monofilaments using the up-down method. Rats were placed in individual chambers (23 × 17 × 14 cm) with a wire mesh floor and allowed to acclimatise for 15 min or until cage exploration stopped. The habituation to the testing conditions lasted 3 days. Cutaneous sensitivity was analysed in the lower abdomen and in the right hindpaw, known to be areas of referred pain associated with visceral pathologies (Jarrell, 2009). The hindpaw was chosen instead of the inner thigh due to difficulty in touching this area with the von Frey filaments. No differences were found between the sensitivity of the right and left hindpaws (data not shown). To determine the abdominal or hindpaw MT, the lower abdominal region and the right hindpaw were touched with one of a series of eight Von Frey monofilaments (rated at 2, 4, 6, 8, 15, 26, 60, and 100 g). Filaments were applied 5 s perpendicularly to the plantar surface with enough strength to cause the monofilament to slightly bend. Each filament was tested five times, with an interval of 30 s between filaments. Testing was initiated with the 2-g monofilament. The remaining filaments were applied in a consecutive fashion. In the case of no response to the filament, the next-stronger monofilament was applied. A positive response was recorded when the animal reacted to the filament (sharp paw withdrawal, licking of the paw or jump) in three out of the five filament applications.

In the first part of this work, regarding the effects of intrathecal BDNF administration, the MTs of the abdominal region and of the right hindpaw were determined after intrathecal injection of saline or 1.5 μg of BDNF. As described above, the injected volume was 20 μl followed by a 20 μl saline flush. Baseline values were determined prior to surgical placement of the intrathecal catheter (acute NT administration) and before catheter placement and attachment of mini-osmotic pumps to the catheter (chronic NT administration). The evaluation of the acute effect of intrathecal injections of saline or BDNF (1.5 μg) in non-inflamed animals was performed for 30 min and initiated immediately after injection. The effect of chronic administration of saline or BDNF was evaluated daily for 5 days. BDNF was delivered at a rate of 1.5 μg per day.

In the second part of this work, regarding the effect of BDNF blockade, the MTs of the lower abdominal region and right hindpaw of the animals were determined before (baseline MT) and after the induction of bladder inflammation. After establishing the baseline MT, animals were injected with CYP. At 4, 24 and 48 h post-CYP injection, animals received intrathecal injections of saline, k252a (2 or 6 μg), and TrkB-Ig$_2$ (1 or 10 μg) in a final volume of 20 μl. Each injection was followed by a 20 μl flush of saline to assure complete drug delivery. Fifteen minutes later, the MT of the lower abdomen and right hindpaw were determined.

2.6 Perfusion and immunocytochemistry

After cystometry or behavioural assessment, animals were perfused through the ascending aorta with cold oxygenated calcium-free Tyrode's solution (0.12 M NaCl, 5.4 mM KCl, 1.6 mM, $MgCl_2.6H_2O$, 0.4 mM $MgSO_4.7H_2O$, 1.2 mM $NaH_2PO_4.H_2O$, 5.5 mM glucose, 26.2 mM $NaHCO_3$), followed by 4% buffered paraformaldehyde. The dissection of the perfused nervous tissue allowed the confirmation of the position of the intrathecal catheter. The spinal cord segments L5-L6 were collected, post-fixed for 4 h and cryoprotected for 24 h in 30% sucrose with 0.1% sodium azide in 0.1 M phosphate buffer.

Transverse 40-μm sections of the collected spinal cord segments were cut on a freezing microtome and stored in cryoprotective solution at −20 °C until tested for phosphoERK immunoreactivity. After inhibition of endogenous peroxidase activity and thorough washes in PBS and PBST, sections were incubated in 10% normal swine serum in PBST for 2 h. Sections were then incubated for 48 h at 4 °C with a specific antibody against phosphoERK1/2 (1:1000). Subsequently, sections were washed and incubated with polyclonal swine anti-rabbit biotin conjugated antibody (1:200). In order to visualize the immunoreactions, the ABC conjugated with peroxidase (1:200) method was used with the chromogen 3,3-diaminobenzidine-tetrahydrochloride (DAB; 5 min in 0.05 M Tris buffer, pH 7.4 containing 0.05% DAB and 0.003% hydrogen peroxide). Sections were mounted on gelatine-coated slices and air-dried for 12 h, cleared in xylene, mounted with *Eukitt* mounting medium and cover-slipped.

BDNF immunoreactivity was also measured in spinal sections from CYP-inflamed animals receiving saline or TrkB-Ig2. Cryoprotected cord sections were thoroughly washed in PBS and PBST. Sections were incubated in 10% normal goat serum in PBST for 2 h, after which they were incubated for 48 h at 4 °C in anti-BDNF antibody (1:1000). Sections were then washed in PBST and incubated for 1 h in Alexa-fluor 488 goat anti-rabbit (1:2000). After this, sections were mounted in Vectashield mounting medium and observed using a Zeiss microscope (Axioimager Z1). Eight random transverse sections per animal were selected for analysis. The software used for analysis was Fiji Software (based on ImageJ, http://rsb.info.nih.gov/ijJava1.6.0_20, 32 bit) to determine the average intensity of BDNF immunofluorescence within the dorsal horns (DH). Background intensity was deducted from the average intensity to calculate the mean net staining intensity.

To control for specificity of phosphoERK and BDNF immunoreactions, spinal sections were incubated in PBST in the same conditions (4 °C, 48 h) in the absence of the respective antibodies. No positive staining was observed under these conditions.

2.7 Quantification and statistics

Cystometrograms were analysed using Data Trax software (version 1.804; World precision Instruments). The frequency of bladder contractions, peak pressure, amplitude of bladder contraction and AUC were analysed by using Kruskal–Wallis One-Way repeated measures analysis of varience (ANOVA) in SigmaStat software. Data are presented as mean value ± standard deviation (SD) and $p < 0.05$ was considered to be statistically significant.

The behavioural data obtained following intrathecal BDNF administration, in the presence or absence of k252a or TrkB-Ig_2 were analysed using One-Way repeated measures ANOVA, which was followed by the post hoc Student–Newman–Keuls test using SigmaStat 3.11 software. In all cases, $p < 0.05$ was considered to be statistically significant. Data are presented as the mean ± SD.

PhosphoERK1/2 expression was analysed in spinal sections from CYP-inflamed animals that received intrathecal saline, k252a or TkB-Ig_2 and had also been assessed for behavioural changes. The number of phosphoERK immunoreactive (IR)-cells was counted in the DH, dorsal commissure (DCM) and intermediolateral grey matter areas of the cord (ILGs) in 10 non-contiguous spinal cord sections. In this case, statistical analysis was performed using One-Way ANOVA followed by the post hoc Student–Newman-Keuls test, using SigmaStat 3.11 software. Data are presented as mean number of IR-cells per spinal cord section ± SD and $p < 0.05$ was considered statistically significant.

The intensity of BDNF immunofluorescence in rats receiving saline of TrkB-Ig_2 was compared using One-Way ANOVA, followed by the post hoc Student–Newman–Keuls test in SigmaStat 3.11 software. $p < 0.05$ was considered to be statistically significant.

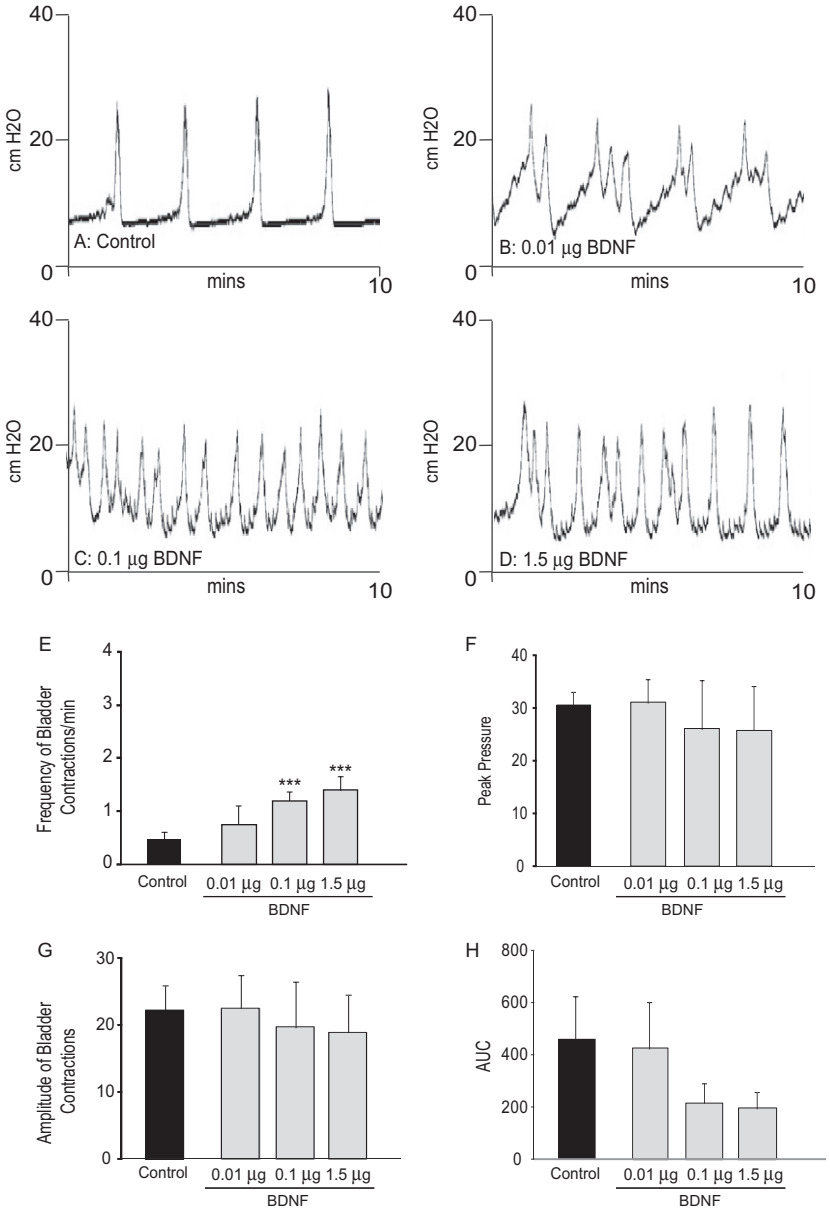

Fig. 1. (A–H) Representative cystometrograms of control non-inflamed animals receiving acute sequential injections of BDNF (0.01, 0.1 and 1.5 µg). (A–D) In the group of animals receiving BDNF, a dose-dependent increase in bladder reflex activity was observed. (E–H) Histograms showing the mean frequency (E), peak pressure (F) and amplitude (G) of bladder contractions, and area under the curve (AUC; H) of non-inflamed animals treated with BDNF. (E) Acute intrathecal injection of 0.1 and 1.5 µg of BDNF significantly increased the frequency of bladder contractions (***$p < 0.001$ versus control animals). (F–H) The amplitude of bladder contractions, peak pressure and AUC remained unchanged in non-inflamed animals treated with BDNF.

2.8 Haematoxylin-eosin staining procedures

The presence of inflammation was assessed in non-inflamed animals (n = 4) and in all animals receiving saline or CYP using haematoxylin–eosin staining to assess bladder histology. Bladders were fixed overnight in 10% buffered formalin solution and blocked in paraffin on the following day. Bladder tissue was sectioned into 6-μm sections. Sections were then stained with haematoxylin and eosin.

RESULTS

Exogenous administration of BDNF

Bladder reflex activity after intrathecal administration of BDNF to non-inflamed rats. To evaluate the acute effect of BDNF on bladder reflex activity, non-inflamed rats received sequential intrathecal injections of increasingly greater amounts of BDNF, in a total of three injections per animal. At baseline, the frequency of bladder contractions of control animals was 0.46 ± 0.1 contraction/min (Fig. 1A, E). Administration of the lowest dose of BDNF (0.01 μg) resulted in a non-significant increase to (0.75 ± 0.4 contractions/min; Fig. 1B, E). After intrathecal treatment with 0.1 μg and 1.5 μg of BDNF, the frequency of bladder contractions significantly increased to 1.20 ± 0.2 and 1.40 ± 0.3 contractions/minute, respectively (p < 0.001 versus control animals; Fig. 1C–E). This increased frequency of bladder contractions was only observed in the 10-min period following BDNF administration, after which the frequency of bladder contractions returned to the baseline. Changes in the frequency of bladder reflex were not accompanied by alterations in the peak pressure, amplitudes of bladder contractions and AUC (respectively, Fig. 1F–H).

The effect of chronic intrathecal administration of BDNF on bladder function was studied by using mini-osmotic pumps for chronic delivery of sterile saline or BDNF. BDNF was administered at a rate of 1 μl/h (1.5 μg/day) for 5 days. Measurements were taken on the sixth day. In animals receiving chronic saline, the frequency of bladder contractions was 0.38 ± 0.1 contractions/minute (Fig. 2A, C), similar to that observed in control non-manipulated rats. Chronic intrathecal BDNF treatment did not significantly alter the frequency of bladder contractions (0.47 ± 0.1 contractions/min; Fig. 2B, C). As observed following acute intrathecal BDNF injection, no changes were observed in the peak

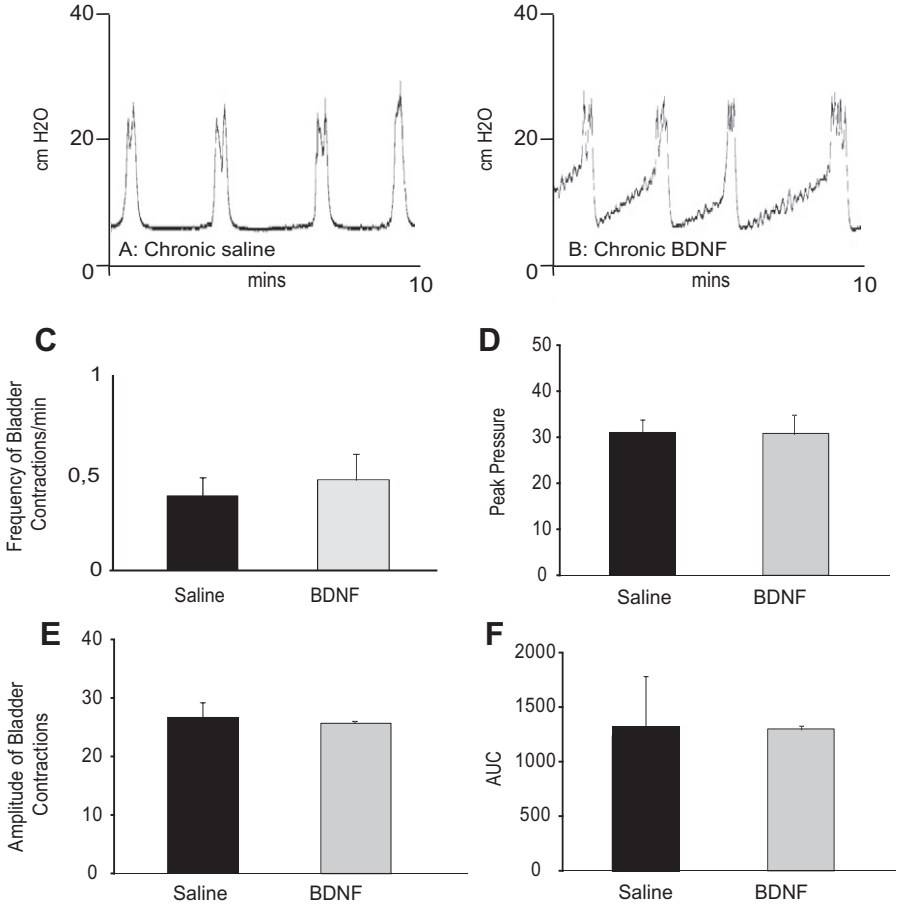

Fig. 2. (A, B) Representative cystometrograms of non-inflamed animals receiving chronic intrathecal infusion of saline or BDNF (1.5 μg/day). (A) Bladder function of non-inflamed animals receiving chronic saline was not affected. (B) Chronic treatment with BDNF did not produce any effect. (C–F) Histograms showing the mean frequency (± SD) of bladder contractions of non-inflamed animals treated with chronic saline or BDNF. Saline and BDNF-treated animals showed no changes in the frequency (C), peak pressure (D) and amplitude (E) of bladder contractions, and AUC (F).

pressure, amplitudes of bladder contractions and AUC (respectively, Fig. 2D–F).

Cutaneous sensitivity after acute and chronic intrathecal administration of BDNF to non-inflamed rats. In this set of experiments, the effects of acute BDNF administration on animal behaviour were evaluated immediately after intrathecal injection of saline and 1.5 μg of BDNF. In control non-inflamed animals treated with saline, the basal MT on the abdominal region was 60.00 ± 0.2 g, remaining constant throughout the period of testing (Fig. 3A). The group of animals treated with BDNF presented with a basal abdominal MT of 48.60 ± 15.1 g (Fig. 3A). Acute intrathecal administration of 1.5 μg of BDNF caused a significant reduction ($p < 0.001$ between 4 and 19 min and $p < 0.05$ 22–24 min versus non-inflamed animals treated with saline; Fig. 3A). The minimum value (7.30 ± 0.9 g) was observed 12 min after BDNF administration (Fig. 3A). After this time point, the abdominal MT returned to baseline values.

On the right hindpaw, the basal MT of control animals treated with saline was 26.00 ± 0.3 g and remained at similar values throughout the whole testing period (Fig. 3B). In the group of animals that received 1.5 μg intrathecal BDNF the baseline hindpaw MT was 26.00 ± 0.5 g (Fig. 3B). Intrathecal BDNF administration resulted in a significant ($p < 0.05$ versus control animals treated with saline) reduction of MT in the hindpaw; in all animals the minimum value was observed 17 min after BDNF administration (10.00 ± 0.0 g). After the 22-min time point, the MT increased again to baseline values.

The effects of chronic intrathecal treatment of BDNF on cutaneous sensitivity were also assessed. In the group of animals administered saline chronically, the abdominal baseline MT was 60.00 ± 0.2 g (Fig. 4A). A decrease of the MT to 15.00 ± 0.1 g was observed, at 24 h post-CYP possibly due to the subcutaneous placement of the osmotic mini-pump. The abdominal MT increased to 26.00 ± 0.0 g at the remaining time points. In the group of animals receiving chronic BDNF (1 μl/h; 1.5 μg/day), the baseline abdominal MT on the lower

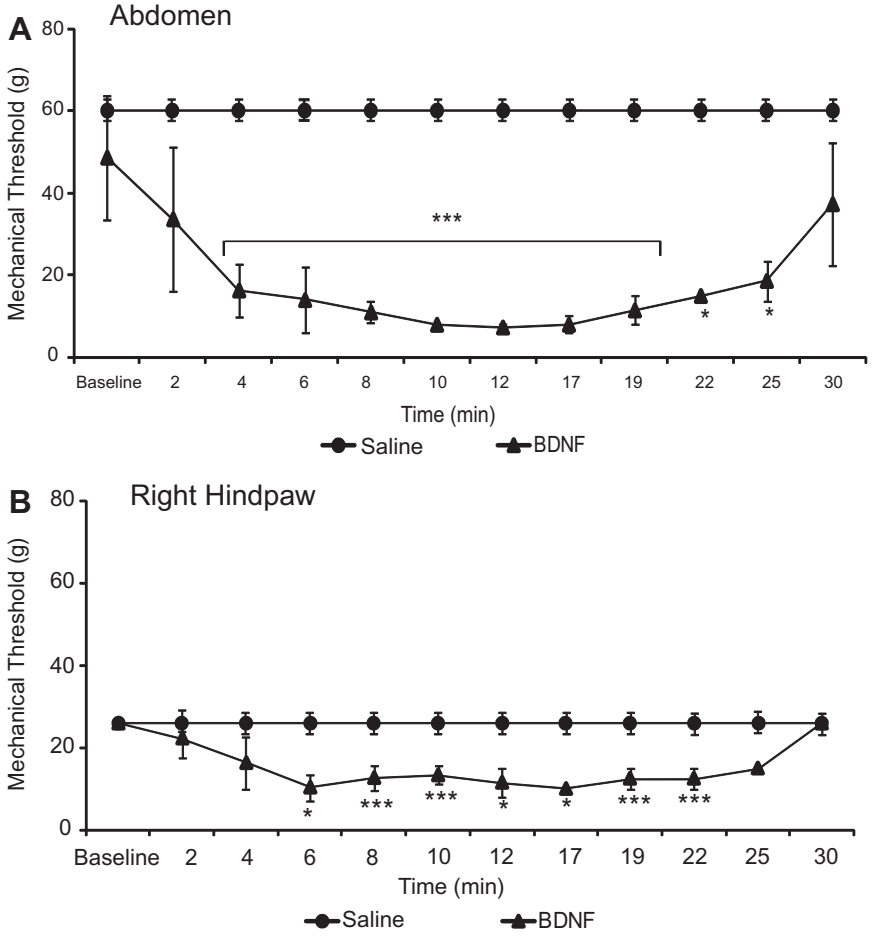

Fig. 3. Mechanical thresholds (MTs) of the lower abdomen (A) and right hindpaw (B) from non-inflamed animals after acute intrathecal administration of saline and BDNF (1.5 μg). (A) In the lower abdomen of non-inflamed animals receiving saline, the MTs remained constant throughout the testing period. The group of animals treated with BDNF presented a significant reduction of the abdominal MT between the 4th and 25th min (4th-19th min $^{***}p < 0.001$; 22nd–24th min $^{*}p < 0.05$ versus saline-treated animals). (B) In the right hindpaw, the MT of saline-treated animals was changed by the intrathecal treatment. The group of animals receiving BDNF showed a reduction in the MT between the 6th and 22nd min ($^{*}p < 0.05$ or $^{***}p < 0.001$ versus saline-treated animals). For some points, error bars (± SD) are not visible as there was no variation in values between animals.

abdomen was 60.00 ± 0.1 g (Fig. 4A). The abdominal MT significantly decreased 24 h after intrathecal placement of the osmotic pump and remained similarly low until the end of the behavioural assessment. The MT values registered were 9.50 ± 5.5 g at 24 h, 6.00 ± 2.0 g at 48 h ($p < 0.05$ versus saline-treated animals), 6.00 ± 0.0 g at 72 h and 6.00 ± 0.0 g at 96 h ($p < 0.001$ versus saline-treated animals; Fig. 4A).

In the right hindpaw, the baseline MT of animals receiving chronic saline was 26.00 ± 0.1 g (Fig. 4B). In this group of animals, the hindpaw MT decreased to 15.00 ± 0.1 g at 24 h post-CYP and remained constant at all time points thereafter (Fig. 4B). Chronic administration of BDNF also resulted in a reduction of MT in the hindpaw at all time points of the experiment. The MT significantly decreased from 26.00 ± 0.1 g at baseline to 17.00 ± 12.2 g at 24 h, 10.50 ± 6.4 g at 48 h, 6.00 ± 0.0 g at 72 h ($p < 0.001$ versus saline-treated animals) and 7.00 ± 1.4 g at 96 h ($p < 0.05$ versus saline-treated animals; Fig. 4B).

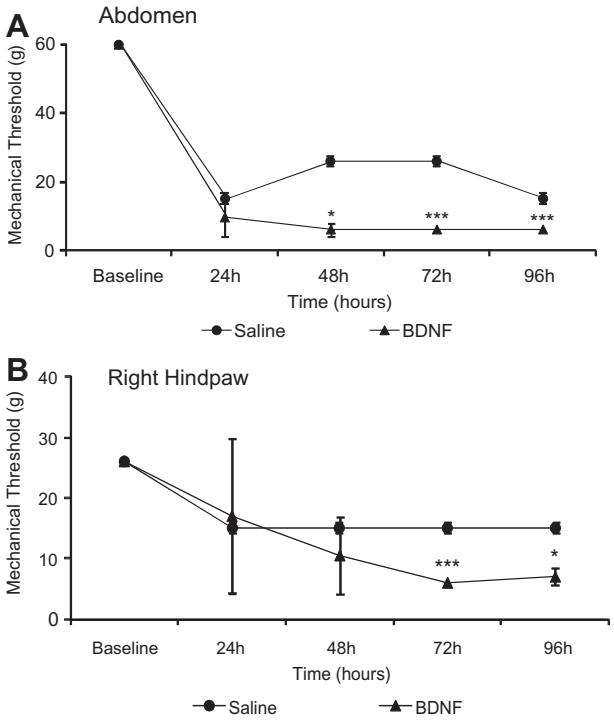

Fig. 4. Mechanical thresholds (MTs) of the lower abdomen (A) and right hindpaw (B) from non-inflamed animals after chronic intrathecal infusion of saline and BDNF (1.5 μg/day) during 5 days. (A) In the lower abdomen, saline-treated animals presented a decrease in the MT 24 h after placement of the osmotic pump, increasing at later time points. In the group of animals receiving chronic BDNF, the MT was significantly reduced at all experiment time-points in comparison with saline-treated animals ($^{*}p < 0.05$ at 48 h; $^{***}p < 0.001$) at 72–96 h. (B) In the right hindpaw, the MT of non-inflamed animals treated with saline remained unaltered. Chronic infusion of BDNF lead to a significant reduction in the MT only at 72 h ($^{*}p < 0.001$) and 96 h of treatment ($^{*}p < 0.05$) when compared to saline-treated animals. For some points, error bars (±SD) are not visible as there was no variation in values between animals.

Blockade of Trk receptors and BDNF sequestration

As exogenous administration of BDNF resulted in heightened bladder reflex activity and increased cutaneous sensitivity, we sought to investigate if BDNF blockade would improve bladder hyperactivity and referred pain in rats with cystitis. For that, we used k252a, a general blocker of Trk receptors, and TrkB-Ig_2, a BDNF scavenger (Banfield et al., 2001; Naylor et al., 2002).

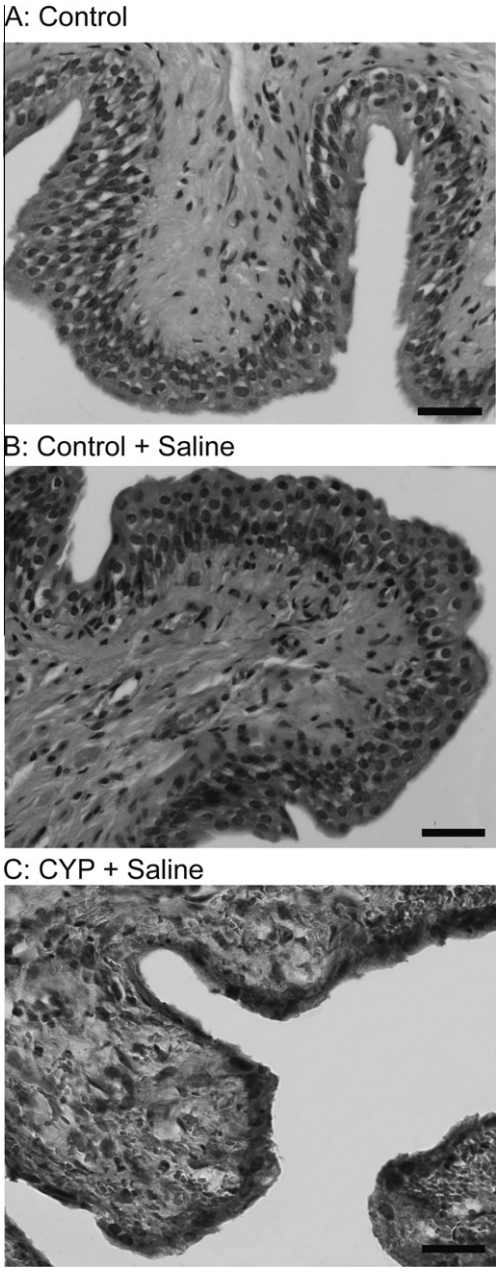

Fig. 5. (A–C) Bladder histology of control non-inflamed and CYP-inflamed animals treated with saline. Scale bar = 20 μm. (A, B) Bladder tissue of non-inflamed animals was unchanged after saline treatment. (C) In contrast, bladders from CYP-inflamed animals treated with saline presented obvious signs of inflammation, blood infiltration and oedema in the lamina propria and a reduced thickness of the urothelium.

Bladder histology. Sections from the bladder of CYP-treated animals stained with haematoxilin and eosin showed obvious signs of inflammation (Fig. 5C), in comparison with bladders from non-inflamed (Fig. 5A) and CYP-inflamed rats treated with saline (Fig. 5B). Inflammation signs observed included oedema and blood infiltration in the lamina propria as well as reduced thickness of the urothelium.

Bladder reflex activity after intrathecal injection of saline, k252a and TrkB-Ig$_2$ in CYP-inflamed rats. To explore if BDNF blockade would improve bladder function in chronic bladder inflammation, animals with chronic CYP-induced cystitis received intrathecal saline, k252a or TrkB-Ig$_2$ whilst bladder reflex activity was registered. Increasingly greater amounts of k252a or TrkB-Ig$_2$ were injected every 30 min. K252a was used as an comparison for TrkB-Ig2-mediated effects on bladder function and behaviour. At baseline, the frequency of bladder contractions from CYP-inflamed animals was 0.96 ± 0.1 contractions/minute (Figs. 6B and 7A), a value significantly higher than that observed in non-inflamed control animals (0.50 ± 0.1 contractions/minute; $p < 0.05$; Figs. 6A and 7A). The treatment with intrathecal saline did not alter bladder frequency, which remained at 1.00 ± 0.5 contractions/minute (Figs. 6C and 7A). After intrathecal administration of k252a, the frequency of bladder contractions was significantly reduced to 0.60 ± 0.4 (after 2 μg; $p < 0.05$ versus saline; Figs. 6D and 7A) and 0.66 ± 0.4 contractions/minute (after 6 μg; $p < 0.05$ versus saline; Figs. 6E and 7A).

The frequency of bladder contractions in CYP-inflamed animals treated with TrkB-Ig$_2$ was also reduced following intrathecal administration of 1 and 10 μg of TrkB-Ig$_2$, respectively being 0.40 ± 0.1 and 0.30 ± 0.1 contractions/minute ($p < 0.05$ versus saline; Figs. 6F, G, and 7A). No changes in the peak pressure and amplitudes of bladder contractions and AUC occurred after intrathecal administration of k252a or TrkB-Ig$_2$ (Fig. 7B–D).

Referred pain after intrathecal injection of saline, k252a and TrkB-Ig$_2$ in CYP-inflamed rats. The group of CYP-inflamed animals receiving intrathecal saline presented a significant decrease on the abdominal MT at 4, 24 and 48 h post-CYP injection when compared to values registered before the induction of bladder inflammation (Figs. 8A and 9A). Intrathecal administration of 2 or 6 μg of k252a caused a significant improvement of

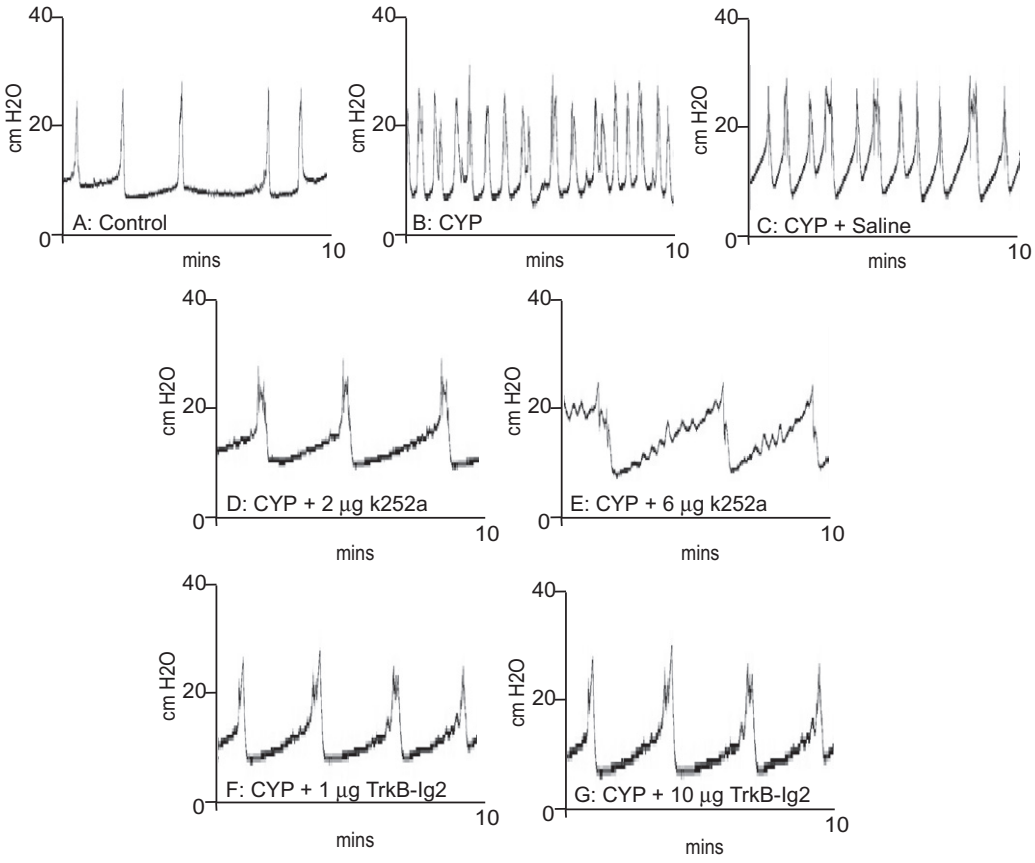

Fig. 6. (A–G) Representative cystometrograms of control and CYP-inflamed animals treated with intrathecal saline, k252a (2 and 6 μg), and TrkB-Ig$_2$ (1 and 10 μg). Saline, k252a, or TrkB-Ig$_2$ was administered via the intrathecal catheter every 30 min during cystometry. (A, B) The basal bladder reflex activity of CYP-inflamed animals was significantly increased when compared to control non-inflamed animals. No differences were found in the urinary frequency of CYP-inflamed animals after intrathecal injection of saline (C). The group of CYP-inflamed rats treated with k252a (D, E) and TrkB-Ig$_2$ (F, G) presented a significant reduction of bladder reflex activity in comparison to CYP-inflamed animals with saline.

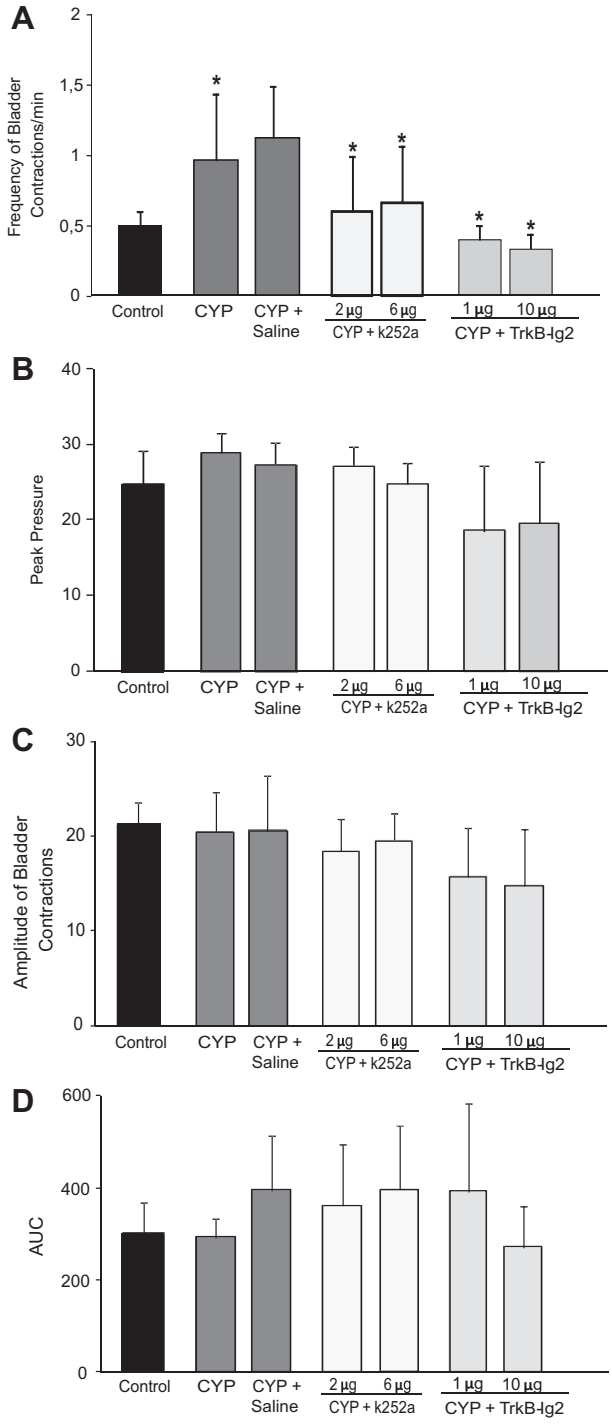

Fig. 7. (A–D) Histograms showing the mean frequency (A), peak pressure (B) and amplitude (C) of bladder contractions and AUC (D) of CYP-inflamed animals treated with saline, k252a (2 and 6 µg), and TrkB-Ig_2 (1 and 10 µg). (A) The frequency of bladder contractions of CYP-inflamed animals receiving saline was significantly elevated when compared to control non-inflamed rats ($^{*}p < 0.05$). No differences were found after intrathecal treatment with saline. The urinary frequency of CYP-inflamed animals was significantly reduced after intrathecal treatment with 2 and 6 µg of k252a ($^{*}p < 0.05$ versus CYP-inflamed animals receiving saline), 1 and 10 µg of TrkB-Ig_2 ($^{*}p < 0.05$ versus CYP-inflamed animals receiving saline). (B–D) The amplitude of bladder contractions, peak pressure and AUC remained unchanged between groups.

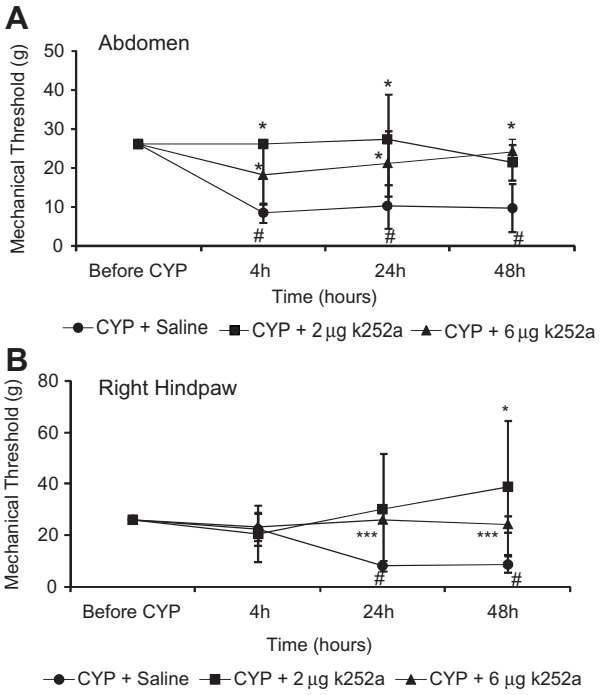

Fig. 8. (A, B) Mechanical thresholds (MTs) of the lower abdomen (A) and right hindpaw (B) of CYP-inflamed animals, injected intrathecally at 4, 24 and 48 h post-CYP injection, with saline and k252a (2 and 6 µg). Saline or k252a was administered 15 min prior to the determination of the MT with the von Frey monofilaments. In the lower abdomen (A), the MT was significantly decreased ($^{*}p < 0.05$ versus before CYP injection) by inflammation and not changed by saline administration. The MT remained low until the end of the experiment. The abdominal MT of CYP-inflamed animals treated with 2 and 6 µg of k252a was significantly increased at 4, 24 and 48 h-post injection ($^{*}p < 0.05$) in comparison to CYP inflamed saline-treated animals. In the right hindpaw (B) the MT decreased only 24 h after CYP administration ($^{\#}p < 0.05$ versus before CYP injection). No differences were found after intrathecal treatment with saline. In the group of inflamed animals receiving k252a, the abdominal MT was significantly increased at 24 and 48 h post-CYP injection ($^{*}p < 0.05$ or $^{***}p < 0.001$ versus CYP-inflamed animals with saline). For some points, error bars (±SD) are not visible as there was no variation in values between animals.

the abdominal MT at all time points tested (Fig. 8A). On the right hindpaw, we only observed a significant decrease of the MT 24 h after CYP injection (Fig. 8B). Intrathecal injection of both doses of k252a significantly increased the MT in the hindpaw (Fig. 8B).

BDNF sequestration with TrkB-Ig_2 also produced beneficial effects. Indeed, a significant improvement was found both in the abdominal and hindpaw MT following intrathecal injection of the BDNF sequestrant at all time points of inflammation (Fig. 9A, B). However, despite the effects on bladder reflex activity, it should be noted that treatment with 10 µg of TrkB-Ig_2 in awake animals produced significant side-effects, such as the loss of equilibrium. Thus, behavioural assessment was only performed in two animals, after which they were immediately euthanized. No more experiments were conducted using this dose.

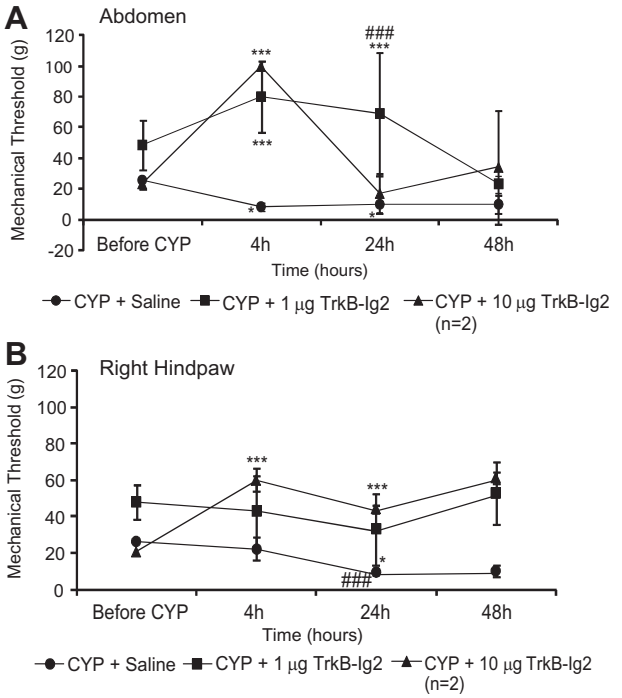

Fig. 9. (A, B) Mechanical thresholds (MTs) of the lower abdomen and right hindpaw of CYP-inflamed animals intrathecally injected at 4, 24 and 48 h post-CYP injection, with saline and TrkB-Ig$_2$ (1 and 10 μg). Saline or TrkB-Ig2 was administered via the intrathecal catheter every 30 min during cystometry, and 15 min prior to the determination of the MT with the von Frey monofilaments. In the lower abdomen (A), the MT of CYP-inflamed animals receiving saline was significantly reduced throughout the experiment (*$p < 0.05$ versus before CYP injection). The abdominal MT of CYP-inflamed animals treated with 1 μg of TrkB-Ig$_2$ was significantly improved at 4 and 24 h post-CYP-injection (***$p < 0.001$ versus CYP-inflamed animals treated with saline). However, the MT of inflamed animals treated with 10 μg of TrkB-Ig$_2$ ($n = 2$) was only improved at 4 h post-CYP injection. In the right hindpaw (B), the MT was significantly improved in CYP-inflamed animals receiving 1 and 10 μg of TrkB-Ig$_2$ (***$p < 0.001$ versus CYP-injected animals treated with saline). For some points, error bars (±SD) are not visible as there was no variation in values between animals. In addition error bars cannot be given for animals receiving 10 μg of TrkB-Ig$_2$ as $n = 2$.

Spinal cord ERK activation after intrathecal injection of saline, k252a and TrkB-Ig$_2$ in CYP-inflamed rats. Immunostaining of L5/L6 spinal cord sections of CYP-inflamed animals treated with saline revealed the presence of phosphoERK-positive cells, distributed bilaterally on the superficial laminae I and II of the DHs, on the DCM and on the ILGs. The number of positive cells observed in the DHs of CYP-inflamed rats receiving saline was 24.70 ± 8.9 (Figs. 10A and 11A). Intrathecal administration of 2 and 6 μg k252a resulted in a decrease to 12.10 ± 4.0 and 7.70 ± 3.6 ($p < 0.05$ versus CYP-inflamed animals treated with saline; Figs. 10B, C and 11A). Likewise, intrathecal injection of 1 μg of TrkB-Ig$_2$ significantly reduced the number of phosphoERK cells to 8.60 ± 1.7 ($p < 0.05$ versus CYP-inflamed animals treated with saline; Figs. 10D and 11A).

In the DCM, the number of phosphoERK-IR cells in spinal sections from CYP-inflamed animals treated with saline was 15.20 ± 0.6 (Figs. 10A and 11B). Following the administration of k252a, the number of positive cells was 12.3 ± 6.2 and 6.40 ± 3.4 after intrathecal injection of, respectively, 2 and 6 μg of the compound ($p < 0.05$ versus CYP-inflamed animals treated with saline; Figs. 10B and 11B). After intrathecal administration of 1 μg of TrkB-Ig$_2$ the number of IR cells was 8.40 ± 3.1 ($p < 0.05$ versus CYP-inflamed animals treated with saline; Figs. 10D and 11B).

In spinal sections of CYP-inflamed rats receiving intrathecal saline, the number of phosphoERK-IR cells observed in the ILGs was 9.30 ± 1.0 (Figs. 10A and 11C). In spinal sections from animals receiving k252a, the number of positive cells was 12.40 ± 3.6 in rats receiving 2 μg and 4.70 ± 3.3 after 6 μg of the drug ($p < 0.05$ versus CYP-inflamed animals treated with saline; Figs. 10B, C, and 11C). In rats receiving 1 μg of TrkB-Ig$_2$ the number of phosphoERK-IR cells was 6.20 ± 0.5($p < 0.05$ versus CYP-inflamed animals treated with saline; Figs. 10D and 11).

BDNF expression at the spinal cord. BDNF was expressed throughout the spinal cord, particularly in the DH (Fig. 12A and B). We found IR cell bodies in all layers of the cord. In the superficial layers of the cord there was also intense immunostaining, most likely reflecting the presence of BDNF in the spinal processes of primary afferents. We found that administration of 1 μg of TrkB-Ig$_2$ resulted in a significant reduction in spinal BDNF expression ($p < 0.05$ versus CYP-inflamed animals, Fig. 12C).

DISCUSSION

In an effort to better understand the role of central effects of BDNF in interstitial cystitis, the present study examined the effects of acute and chronic intrathecal administration of BDNF to non-inflamed rats on bladder function, and BDNF sequestration during CYP-induced bladder inflammation. As in other studies (Pinto et al., 2010), CYP administration induced a pronounced inflammation of the bladder, confirmed by histological analysis of bladder tissue. The results presented here show an increase of urinary frequency immediately after acute intrathecal administration of BDNF, as well as behavioural signs of pain. In addition, animals with chronic cystitis showed a reduction in bladder hyperactivity and referred pain following Trk blockade with k252a or BDNF sequestration with TrkB-Ig$_2$, indicating that this NTs participates in referred pain and bladder hyperactivity in chronic conditions. Surprisingly, following chronic administration of BDNF only allodynia was observed and bladder hyperactivity, observed after acute BDNF administration, was not present.

The effects of acute intrathecal BDNF administration on bladder function and cutaneous sensitivity were observed shortly after injection. The reason for the rapid effect of intrathecal BDNF may be easily understood as being due to the activation of TrkB receptors present in dorsal horn neurons, postsynaptic to bladder afferents. Binding of BDNF to these receptors induces swift downstream activation of intracellular signalling pathways such as the ERK 1 and 2 cascade in spinal neurons postsynaptic to primary afferents (Lever et al.,

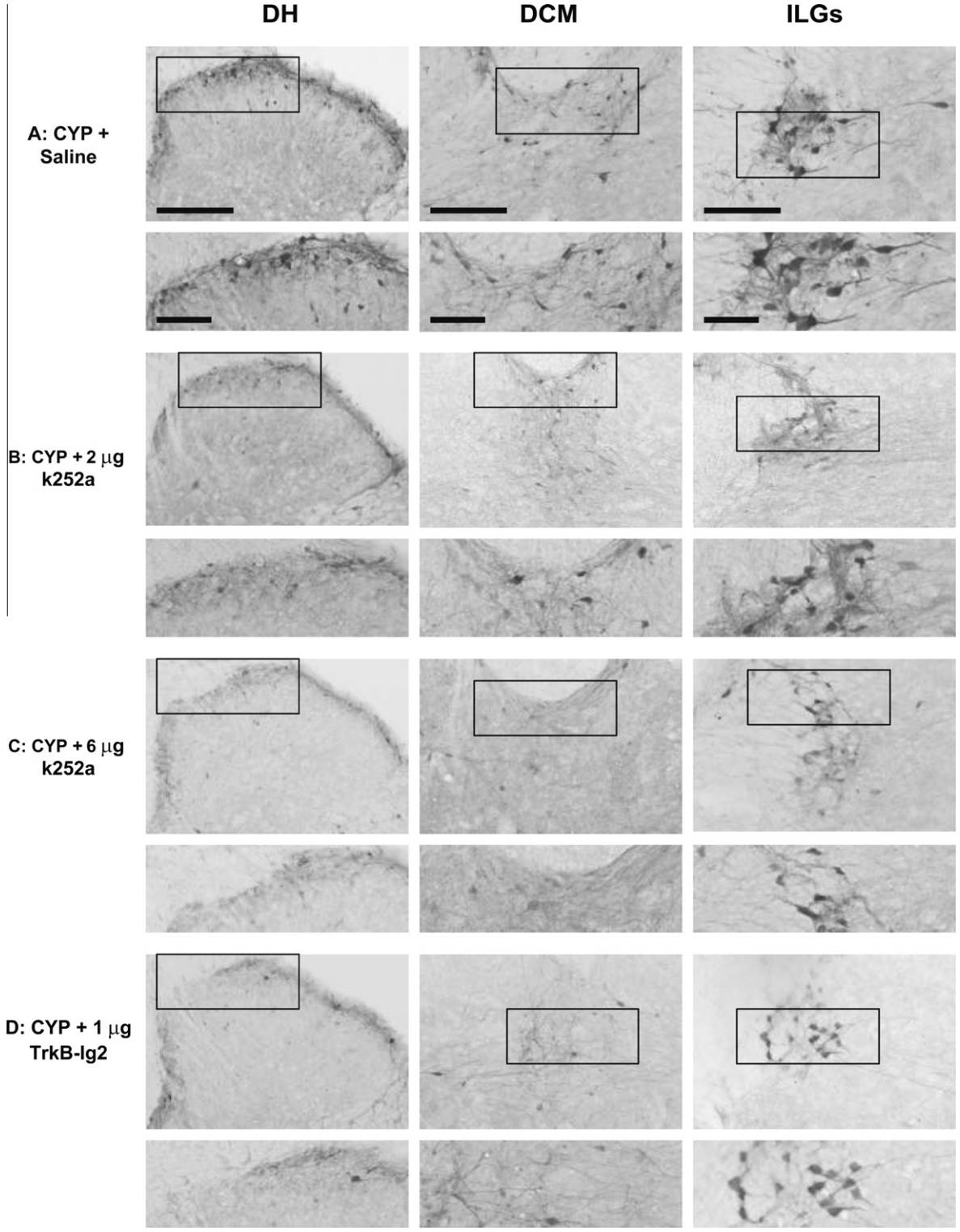

Fig. 10. (A-D) Representative photomicrographs of the total number of phosphoERK-IR cells in L5/L6 spinal cord sections from CYP-inflamed animals after intrathecal injection of saline (A), 2 μg of k252a (B), 6 μg of k252a (C), and 1 μg of TrkB-Ig_2 (D). Scale bars = 100 μm. Magnified images, scale bar = 20 μm. The phosphoERK immunoreactive cells were found in the laminae I and II of the dorsal horn (DH; left column), in the dorsal commissure (DCM; central column) and in the intermediolateral grey matter (ILGs; right column). Trk blockade or NT sequestration resulted in a significant reduction in phosphoERK expression in the analysed areas of the cord. Boxes in images refer to higher power images of phosphoERK-positive cells in the analysed areas of the cord.

2003b; Slack et al., 2004, 2005), an essential mechanism of spinal processing of peripheral noxious input (Ji et al., 1999, 2009; Pezet et al., 2002c; Pezet and McMahon, 2006; Zhao et al., 2006). Also, the activation of this pathway has already been linked to bladder hyperactivity and increased sensory barrage associated

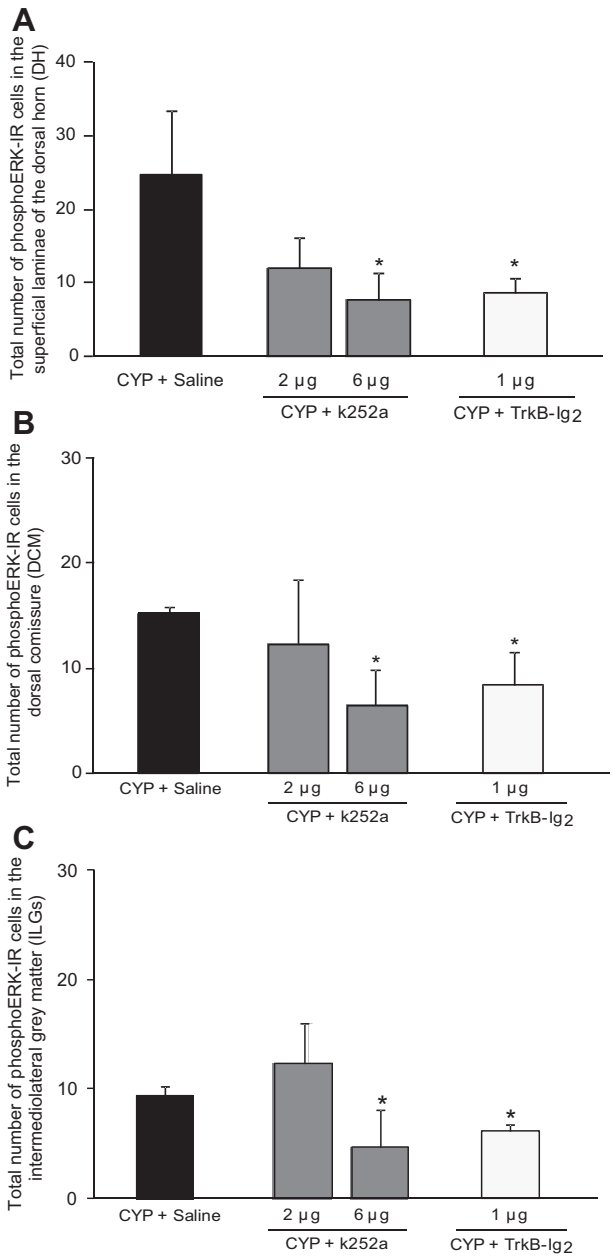

Fig. 11. (A–C) Histograms depicting the mean number (±SD) of phosphoERK activation in dorsal horn (DH), dorsal commissure (DCM) and intermediolateral grey matter (ILGs) in sections from L5/L6 spinal cord. Intrathecal delivery of 6 µg of k252a and 1 µg of TrkB-Ig_2 resulted in significant reduction of phosphoERK expression in the DH, DCM and ILGs in spinal cells ($^*p < 0.05$ versus CYP-inflamed animal treated with saline).

with chronic bladder inflammation (Cruz et al., 2005). In that study, after bladder stimulation we observed a rapid increase in the number of phosphoERK-positive dorsal horn neurons located in the projection areas of bladder afferents (Cruz et al., 2005).

In the present study we observed that the acute effects of intrathecal BDNF were short-lived. Bladder hyperactivity was observed in the 10-min period after BDNF injection. Likewise, allodynia was only observed in the 20-min time frame following intrathecal BDNF administration, after which cutaneous sensitivity returned to baseline values. This is in accordance with previous studies which indicated that the activation of the downstream ERK pathway by BDNF is short-lasting (Pezet et al., 2002b; Lever et al., 2003b; Pezet and McMahon, 2006; Zhao et al., 2006). We have previously demonstrated that in spinal cord neurons, ERK phosphorylation following bladder distension rapidly disappeared 10 min after stimulation and was no longer observed two hours after stimulus (Cruz et al., 2005). The reason for the short duration of the effect of acute BDNF treatment may result from inactivation of the above-mentioned signalling cascade, quickly achieved by specific phosphatases (Muda et al., 1996). In addition, cessation of BDNF signalling in spinal synapses is very likely to be a rapid process, dependent on TrkB internalization, simple diffusion of the NT or its degradation by extracellular proteases (Pezet et al., 2002c; Pezet and McMahon, 2006).

The results observed following acute intrathecal administration of BDNF support the hypothesis that bladder hyperactivity and referred pain in the lower abdomen and hindpaw observed during CYP-induced cystitis may depend on the excessive levels of spinal BDNF. Accordingly, spinal blockade of TrkB receptors with intrathecal k252a or BDNF sequestration with TrkB-Ig_2 resulted in a marked reduction in bladder hyperactivity and referred pain. Likewise, in rats with colonic inflammation, treatment with an anti-BDNF antibody resulted in a decrease in abdominal pain threshold in response to colonic distension (Delafoy et al., 2006). In our CYP-inflamed rats, reduction of bladder hyperactivity and referred pain levels, obtained with k252a or TrkB-Ig2 administration, was accompanied by reduced levels of spinal expression of phosphoERK, indicating reduced BDNF-dependent spinal cord activation. BDNF immunostaining confirmed the effectiveness of the TrkB-Ig_2 scavenging treatment, as reduction of phosphoERK expression was accompanied by a pronounced decrease in spinal BDNF.

In agreement with the considerations above, chronic BDNF induced referred pain, as indicated by signs of allodynia in the lower abdomen and hindpaw. Allodynia is a hallmark of chronic pain and reflects the occurrence of central sensitization (Latremoliere and Woolf, 2009; Woolf, 2011). Activation of spinal TrkB by BDNF is an important event for central sensitization (Ren and Dubner, 2007). In fact, binding of BDNF to TrkB induces the phosphorylation of specific *N*-methyl-D-aspartate (NMDA) subunits via ERK activation (Slack and Thompson, 2002; Slack et al., 2004), an essential mechanism of central sensitization related to chronic somatic (Costigan and Woolf, 2000; Haddad, 2005) and visceral pain (Tian et al., 2008).

Most unexpectedly, chronic BDNF did not produce detectable changes in bladder reflex activity. This was a systematic finding, observed in all animals receiving chronic intrathecal BDNF. A clear explanation can only be speculated upon. One could hypothesize that exogenous BDNF, being a recombinant protein, is metabolized more rapidly than its endogenous

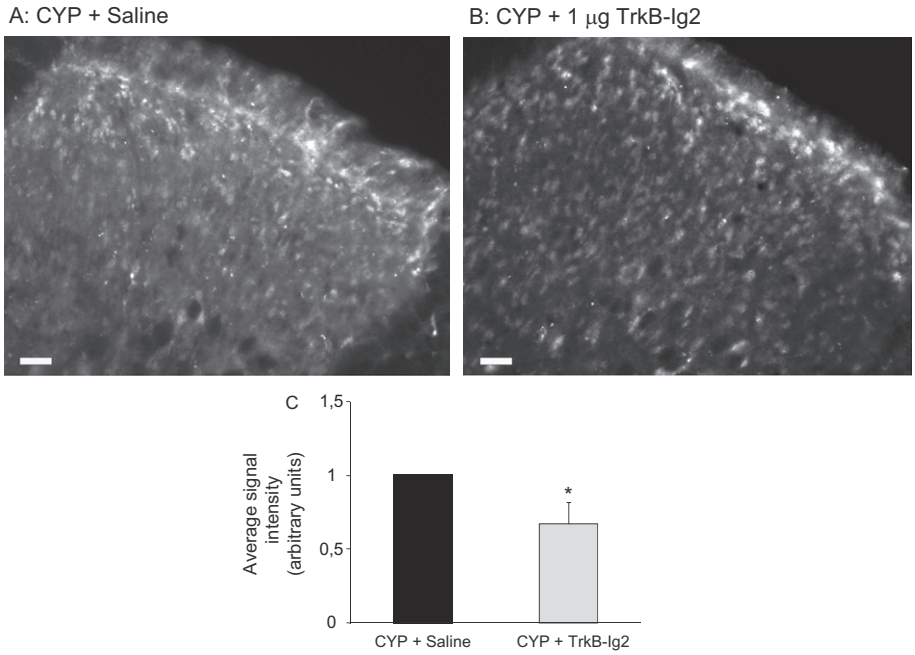

Fig. 12. (A, B) Photomicrographs depicting BDNF expression in the L6 spinal cord segment of CYP-inflamed animals and CYP-inflamed animals treated with TrkB-Ig_2. Scale bar = 20 μm. In the spinal cord, BDNF expression was present in cell bodies throughout the dorsal horn. Immunoreaction was more intense in laminae I and II, reflecting the presence of primary afferents containing BDNF. (C) Graph of bars showing the mean intensity of BDNF expression in the L6 spinal cord segment. BDNF expression was significantly reduced throughout the cord after intrathecal administration of TrkB-Ig_2 ($^*p < 0.05$ versus CYP-inflamed animals treated with saline).

counterpart. However, this explanation is difficult to support by the signs of referred pain, which were similar in rats after chronic BDNF administration or with chronic cystitis which is accompanied by high BDNF levels (Vizzard, 2000). An important aspect to consider is that in the group of rats receiving chronic BDNF treatment the peripheral inflammatory component was absent. This could be essential for the establishment of bladder hyperactivity, indicating a strong dependence on peripheral mechanisms. In addition, one can hypothesize that chronic exposure to BDNF may have enhanced spinal GABAergic interneurons that express TrkB (Pezet et al., 2002a; Lever et al., 2003a; Bardoni et al., 2007). Bladder function is regulated by, and sensitive to, pontine projections that contact with GABAergic spinal neurons (Blok et al., 1997). In fact, potentiation of GABAergic neurotransmission at the spinal cord level is currently viewed as a potential therapeutic solution for neurogenic detrusor overactivity (Miyazato et al., 2008, 2009).

CONCLUSION

The present study highlights the distinctive contribution of BDNF to referred pain and bladder hyperactivity accompanying cystitis. Our experiments with k252a and TrkB-Ig_2, the recombinant BDNF scavenger, suggest that treatment of chronic cystitis could at some point include the modulation of BDNF. Significantly, together with a previous study from our group (Pinto et al., 2010), the present results support a novel role for BDNF in chronic bladder inflammation.

Acknowledgments—The authors would like to acknowledge Professor António Avelino and Dr. Jorge Ferreira for helpful discussions and critical reading of the manuscript. This work has been funded by project INComb FP7 HEALTH Project No. 223234 and by Associação Portuguesa de Urologia (Portuguese Association of Urology). Bárbara Frias is supported by a PhD scholarship SFRH/BD/63225/2009 from FCT (Fundação para a Ciência e Tecnologia), Portugal. Dr Shelley Allen is supported by the Medical Research Council UK and SARTRE – Severnside Alliance Translational Research Enterprise).

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(Accepted 19 December 2012)
(Available online 8 January 2013)

Publication IV

The role of Brain Derived Neurotrophic Factor (BDNF) in the development of neurogenic detrusor overactivity (NDO)

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Manuscript submitted

The role of Brain Derived Neurotrophic Factor (BDNF) in the development of neurogenic detrusor overactivity (NDO)

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[M] 5ŜLJI·NÚƳŜyÚ 2Ŧ 9ÈLJŜNƳŜyÚI·f . ƛ2f2∃ëΣ CI·Õdz tÚè 2Ŧ a ŜŘƛÕIyŸŜ 2Ŧ t2NÚ2Σ ¦ yÍØŜNăÚê 2Ŧ t2NÚ2Σ t2NÚdz∃I·fΠ L . a / - LyãÚÚdzÚ2 ŘŜ . ƛ2f2∃I· a 2fŜÕdzfI·NΩ Ŝ / Ŝfdzf I·NΩ ¦ yÍØŜNăÚŘI·ŘŜ Ř2 t2NÚ2Σ t2NÚdz∃I·fΠ [O] a 2fŜÕdzfI·NJ bŜdzN2ÕƛI2f2∃ë ¦ yƛÚΣ ¦ yÍØŜNăÚê 2Ŧ . NăÚ2fΣ {ÕK22f 2Ŧ / ŒyƛÕI·f {ÕƛŜyÕŜăΣ 52N2ÚKè I 2ŘƎ]ƛy . dzƛfŘƛyƎ∃Σ . NăÚ2fΣ ¦ YT[n] 5ŜLJI·NÚƳŜyÚ 2Ŧ ¦ N2f2∃ëΣ I 2ăLJƛÚI·f ŘŜ {Φ W2ń2Σ t2NÚ2Σ t2NÚdz∃I·fΦ

Abstract bŜdzN2∃ŜyƛÕ ŘŜÚNdză2N 2ØŜNI·ÕÚMÚê ób5hú ƛă I· ǿŜff-]y2ǿy Õ2yãŜIjdzŜyÕŜ 2Ŧ ăLJƛyI·f Õ2NŘ ƛy?dzNΩè ó{ /Lú ÚKI·ú ŜY ŜN∃Ŝă I·ŦúŜN I· LJŜNΩ2Ř 2Ŧ ăLJƛyI·f ăK2Õ] ŘdzNΩyƎ ǿKƛÕK ÚKŜ ōfI·ŘŘŜN ƛă I·NŜŦfŜÈƛÕ ¢KŜ I·LJLJŜI·NJ·yÕŜ I·yŘ YI·ƛyÚŜyI·yÕŜ 2Ŧ b5h I·NŜ ŘŜLJŜyŘŜyÚ 2y LJN2Ŧ2dzyŘ LJfI·ăÚƛÕ ÕKI·y∃Ša 2Ŧ ÚKŜ ăLJƛyI·f ySdzN2yI·f LJI·ÚKǿI·èă ÚKI·ú NŜ∃dzfI·ÚŜ ōfI·ŘŘŜN Ŧdzy ÕÚƛ2y¢ Lú ƛă ∃ŜySNJ·fTè I·ăădzYŜŘ ÚKI·ú ySdzN2ÚN2LJKƛyã ób¢áú I·NŜ YI·ƨ2NJ NŜ∃dzfI·ú2Nă 2Ŧ ădzÕK ySdzN2LJfI·ăÚƛÕ ÕKI·y∃Ŝă¢ bŜNØŜ ∃N2ǿÚK ŦI·ÕÚ2NJ óbDCú ƛă ÚKŜ õŜăÚ ăÚdzŘƛŜŘ b¢ ƛYÚKŜ ōfI·ŘŘŜN I·yŘ ƛÚă N2fŜ 2Ŧ bDC ƛy b5h KI·ă I·fNŜI·Ře õŜŜy ŜăÚI·õfƛăKŜŘΦ ! y2ÚKŜN ØŜNè I·ōdzyŘI·yÚ ySdzNΩÚN2LJKƛy ƛă ÕNJI·ƛY ŘŜNƛØŜŘ ySdzN2ÚN2LJKƛÕ ŦI·ÕÚ2N ó . 5bCú¢ ! fÚK2dz∃K ƛÚ KI·ă õŜŜy NŜÕŜyÚfè ăK2ǿy ÚKI·ú . 5bCΣ I·ÕÚƛyƎ I·ú ÚKŜ ăLJƛyI·f Õ2NŘ fŜØŜfΣ ƛă I·]Ŝè YŜŘƛI·ú2N 2Ŧ õfI·ŘŘŜN Řèă Ŧdzy ÕÚƛ2y I·yŘ LJI·ƛy ŘdzNΩyƎ Õèăú ÚƛăΣ ÚÚ ƛă LJNŜăŜyÚfè dzyÕfŜI·NJ ƛŦ . 5bC ƛă I·fă2 ƛYLJ2NÚI·yÚ Ŧ2N b5h¢ ¢KŜNŜŦ2NŜΣ ÚKŜ LJNŜăŜyÚ ăÚdzŘè I·ƛYŜŘ ú2 ÕfI·NΛŦè ÚKƛă ƛădzŜ¢

wŜădzfÚă 2õÚI·ƛySŘ LJƛyLJ2ƛyÚ . 5bC I·ă I·y ƛYLJ2NÚI·yÚ NŜ∃dzfI·ú2N 2Ŧ b5h ŜYŜN∃ŜyÕŜ I·yŘ YI·ƛyÚŜyI·yÕŜ¢ Ly NJI·úă ǿƛÚK ÕKN2yÑÕ {/LΣ . 5bC ăŜIjdzŜăÚNJI·Úƛ2y ƛYLJN2ǿŜŘ ōfI·ŘŘŜN Ŧdzy ÕÚƛ2yΣ ădz∃∃ŜăÚƛyƎ ÚKI·ÚΣ I·ú fI·ÚŜN ăÚI·∃ŜăΣ . 5bC Õ2yÚNΛõdzÚŜă ú2 ÚKŜ YI·ƛyÚŜyI·yÕŜ 2Ŧ õfI·ŘŘŜN ŘèăŦdzyÕÚƛ2y¢ 5dzNΛyƎ ăLJƛyI·f ăK2Õ] . 5bC yŜ∃I·ƛǿŜfè Y2ŘdzfI·ÚŜă ∃N2ǿÚK 2Ŧ ăŜyă2NΩè I·ŦŦŜNŜyÚăΣ NŜLJNŜăădzNŜ ySdzN2ÚN2LJKƛyã õfI·ŘŘŜN KèLJŜNJI·ÕÚƛØÚê¢ ! ÕÕ2NΩŘƛyƎfèΣ . 5bC ăŜIjdzŜăÚNJI·Úƛ2y NŜădzfÚŜŘ ƛy ƛyÕNŜI·ăŜŘ I·Ë2yI·f ∃N2ǿÚK I·yŘ ŜI·Nfè õfI·ŘŘŜN KèLJŜNJI·ÕÚƛØÚê¢ ¢KŜăŜ ŦƛyŘƛyƎă Kƛ∃KfÚ∃KÚ úKŜ Õ2yÚNΛõdzÚƛ2y 2Ŧ . 5bC ú2 úKŜ ŜYŜN∃ŜyÕŜ 2Ŧ b5h I·yŘ YI·è ƛYLJI·ÕÚ úKŜ ∃ŜyŜNJI·Úƛ2y 2Ŧ Y2NŜ ŜŦŦΛÕƛŜyÚ úKŜNJI·LJŜdzÚƛÕ YŜI·ădzNŜă ú2 YI·yI·∃Ŝ {/L LJI·ÚƛŜyÚă¢

Introduction

hyŜ 2Ŧ úKŜ Y2ăÚ ƛYLJ2NÚI·yÚ Õ2yãŜIjdzŜyÕŜă 2Ŧ ăLJƛyI·f Õ2NŘ ƛy?dzNΩè ó{ /Lú ƛă ăŜØŜNŜ dzNΩyI·NΩè Řèă Ŧdzy ÕÚƛ2y óŘŜ DN2I·ú ² /Σ мффлТ /NdzÎ I·yŘ /NdzÎΣ нлммú¢ ¢NJI·dzYI· ƛă ƛYYŜŘƛI·ÚŜfè Ŧ2ff2ǿŜŘ õè ăLJƛyI·f ăK2Õ]Σ ŘdzNΩyƎ ǿKƛÕK ÚKŜ õfI·ŘŘŜN ƛă I·NNŜŦfŜÈƛÕ I·yŘ dzNΩyI·NΩè NŜÚŜyÚƛ2y 2Õ Õdz NΩă¢ Ly ÚƛYŜΣ ƛy fŜăƛ2yã I·õ2ØŜ ăI·ÕNJI·f ăLJƛyI·f Õ2NŘ ăŜ∃YŜyÚăΣ ăLJƛyI·f ăK2Õ] ƛă 2dzÚǿŜƛ∃KŜŘ õè ÚKŜ ŜYŜN∃ŜyÕŜ 2Ŧ I·y ƛyØ2ŦdzyÚI·NΩè YƛÕÚdzNΩÚƛ2y NŜŦfŜÈ Õ2yÚN2ffŜŘ õè I· ÕƛNÕdzƛÚ ú2ÚI·ffè f2ÕI·ÚŜŘ õŜf2ǿ ÚKŜ I·NŜI· 2Ŧ ÚKŜ fŜăƛ2y¢ ¢Kƛă ySǿ ăLJƛyI·f YƛÕÚdzNΩÚƛ2y NŜŦfŜÈ Ř2Ŝă y2Ú ∃dzI·NJ·yÚŜŜ ÚKŜ Õ22NŘƛyI·Úƛ2y õŜÚǿŜŜy ŘŜÚNdză2NJ Õ2yÚNJI·ÕÚƛ2y I·yŘ õfI·ŘŘŜN 2dzÚfŜÚ NŜfI·È I·Úƛ2y¢

aI·yè {/L LJI·ÚƛŜyÚă I·NŜ ƛyÕ2yÚƛyŜyÚ I·yŘ ǿƛff LJNŜăŜyÚ I· Ŧdzy ÕÚƛ2yI·f õfI·ŘŘŜN 2dzÚfŜÚ 2õăÚNdzÕÚƛ2y ÕI·ffŜŘ ŘŜÚNdză2NJ-ăLJKƛyÕÚŜNJ-ŘèăăèyŜN∃ƛI· ó5{5ú¢ ¢Kƛă fŜI·Ră ú2 f2yƎ LJŜNƛ2Ră 2Ŧ KƛƎK ƛyÚNJI·ØŜăƛÕI·f LJNŜăădzNŜΣ ǿKƛÕK ŜyŘI·yƎŜNJ ÚKŜ dzLJLJŜNJ dzNΩyI·NΩè ÚNJI·ÕÚ I·yŘ NJŜyI·f Ŧdzy ÕÚƛ2y ówI·õÕKŜØăŢèΣ нллсú¢ Ly I·ŘŘÚƛ2yΣ õfI·ŘŘŜN ŦƛffyƎ ăă LJdzyÕÚdzI·ÚŜŘ õè ƛyØ2ŦdzyÚI·NΩè ŘŜÚNdză2NJ Õ2yÚNJI·ÕÚƛ2yãΣ I· Õ2yŘNÚƛ2y ÚŜNJYŜŘ ySdzN2∃ŜyÕ ŘŜÚNdză2NJ 2ØŜNJI·ÕÚƛØƛÚê ób5hú¢ {dzÕK NΛăŜă ƛy ƛyÚNJI·ØŜăƛÕI·f LJNŜăădzNŜ ŦdzNÚKŜNJ ŜyŘI·y∃ŜNJ ÚKŜ dzLJLJŜNJ dzNΩyI·NΩè ÚNJI·ÕÚ I·yŘ ÕI·y ÚNΩ∃∃ŜNJ ŜLJăă2ŘŜă 2Ŧ dzNΩyI·NΩè ƛyÕ2yÚƛySyÕŜ¢

¢KŜNŜŦ2NŜΣ ƛyƛÚƛI·f YI·yI·∃SYŜyÚ 2Ŧ {/L LJI·ÚƛŜyÚă úèLJƛÕI·ffè ƛyÕfdzŘŜă YŜI·ădzNŜă ú2 LJN2ÚŜÕÚ]ƛŘyŜè Ŧdzy ÕÚƛ2y ōè NŜfƛŜØƛy∃ 5{5 I·yŘ NŜŘdzÕƛyƎ LJNŜăădzNŜ¢ hyÕŜ ƛyÚNJI·ØŜăƛÕI·f LJNŜăădzNŜ ăă dzyŘŜNJ Õ2yÚN2f ƛyÕ2yÚƛySyÕŜ ŜYŜN∃ŜăI·ă I· ÕNΛÚƛÕI·f LJ2ƛyÚ ú2 ÚKŜ

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Ijdz I·fÚè 2Ŧ fƛŦŜ 2Ŧ ÚKŜăŜ LJI·ÚƛŜyÚă¢ wŜădzfÚă ŦN2Y I·ăŜNJŜă 2Ŧ ădzNØŜèă I·NŜ ŜØƛŘŜyÚY I·ŦÚŜNJ ƛYLJN2ØƛyƎ Y2ú2NJ Ŧdzy ÕÚƛ2yΣ NŜ∃I·ƛyƛyƎ õfI·ŘŘŜN Õ2yÚN2f ăă 2Ŧ ÚKŜ KƛƎKŜăÚ LJNΛ2NΛÚè Ŧ2NJ {/L LJI·ÚƛŜyÚă óYdzΣ нллсТ CNJŜyÕK Ŝú I·f¢Σ нлмлТ {ƛYLJă2y ŜÚ I·f¢Σ нлмнú¢ ! yÚƛYdzăÕI·NΛyƛÕă ŘNdzƎă I·yŘ 2y1·õ2ÚdzfƛyÚzY ú2ÈƛY ! I·NŜ Õ2YY2yfè dzăŜŘ ú2 NŜŘdzÕŜ b5h I·yŘ ƛyÚNJI·ØŜăƛÕI·f LJNŜăădzNŜ fŜI·ŘƛyƎ õ2úK ú2 dzLJLJŜNJ dzNΛyI·NΩè LJN2úŜÕÚƛ2y I·yŘ Õ2yÚƛySyÕŜ¢ I2ǿŜØŜNΣ ÚKŜăŜ ÚKŜNJI·LJăŜă I·NŜ ySƛÚKŜNJ I·õfŜ ú2 NŜØŜNÚ y2NJ ú2 LJNŜØŜyÚ ÚKŜ YƛÕÚdzNΛÚƛ2y NŜŦfŜÈ I·ú fdzY õ2ăI·ÕNJI·f ăLJƛyI·f Õ2NŘ fŜØŜf¢

LÚ ƛă ÕdzNΩNŜyÚfè I·ădzYŜŘ ÚKI·ú b5h I·yŘ 5{5 NŜădzfÚ ŦN2Y YI·ăăƛØŜ NŜ2NJ∃I·yƛÎI·Úƛ2y 2Ŧ ySdzN2yI·f LJI·ÚKǿI·èă f2ÕI·ÚŜŘ ƛy ÚKŜ fdzY õ2ăI·ÕNJI·f ăLJƛyI·f Õ2NŘ ó±ƛÎÎI·NJŘΣ нллсТ /NdzÎ I·yŘ /NdzÎΣ нлммú¢ I2ǿŜØŜNΣ ÚKŜ ƬƛyŜ Y2fŜÕdzfI·NJ YŜÕKI·yƛaYă I·NŜ dzyÕfŜI·N¢ ! ØI·ÆfI·õfŜ ăÚdzŘƛŜă ădz∃∃ŜăÚ ÚKI·ú ySdzN2ÚN2LJKƛyã YI·è õŜ ƛYLJ2NÚI·yÚ YŜŘƛI·Ú2NJă 2Ŧ ădzÕK LJfI·ăÚƛÕ ÕKI·y∃Ŝă ótŜÎŜÚ I·yŘ aÖaI·K2yΣ нллсТ ±ƛÎÎI·NJŘΣ нллсú¢ ¢Kƛă ăă LJI·NÚƛÕdzfI·NΛfè ŜØƛŘŜyÚ ƛy ǿKI·ú Õ2yÕŜNJyã bŜNØŜ DN2ǿÚK CI·ÕÚ2NJ óbDCúΣ I· ySdzN2ÚN2LJKƛy ÚKI·ú LJfI·èă I·]Ŝè N2fŜ ƛy ÚKŜ NŜ∃dzfI·Úƛ2y 2Ŧ ÚKŜ LJŜNΛLJKŜNJI·f ySNJØ2dzã ăèăÚŜY¢ LYY dzy2ySdzÚNJI·fÎÎI·Úƛ2y 2Ŧ ÚKƛă b¢ KI·ă õŜŜy ăK2ǿy ú2 LJNŜØŜyÚ õfI·ŘŘŜN Řèă Ŧdzy ÕÚƛ2y ƛy {/L NJI·ÚăóI·ăI·]ƛ ŜÚ I·f¢Σ нллнТ {Ŝ]ƛ ŜÚ I·f¢Σ нллпú¢ ¢KŜ Õ2yÚNΛõdzÚƛ2y 2Ŧ 2ÚKŜNJ b¢ăΣ Y2ăÚ y2ÚI·õfè . NJI·ƛy 5ŜNΛØŜŘ bŜdzN2ÚN2LJKÕ CI·ÕÚ2NJ ó. 5bCúΣ ú2 b5h NŜYI·ƛyã LJ22NJfè ƛyØŜăÚƛ∃I·ÚŜŘ¢ ¢KƛăbC ăă ŜÈÚNŜYŜfè I·õdzyŘI·yÚ ƛy ÚKŜ ÕŜyÚNJI·f ySNJØ2dzã ăèăÚŜY ǿKŜNŜ ƛÚ Y2ŘdzfI·ÚŜă ySdzN2LJfI·ăÚƛÕÚè I·Ú ăLJƛyI·f Õ2NŘ fŜØŜf¢ . 5bC KI·ă I·fNŜI·Řè õŜŜy ăK2ǿy ú2 LJI·NÚƛÕƛLJI·ÚŜ ƛy

ŎŝyΩNJl·f ăŝyăƛΩĺƛÎl·ΩĺƛŽyˊ l·ăă2ŏƛl·ΩŝŘ ǿƛΩK ŎKN2yƛŎ LJl·ƛy óaŝNΩƎKƛ ŝΩ l·f¢Σ нллпT tŝÎŝΩ l·yŘ aŎal·K2yΣ нллcT aŝNΩƎKƛ ŝΩ l·f¢Σ нллyú¢ !ŎdzΩŝ ƛyΩNJl·ΩKŝŎl·f l·ŘYˋƛyƛăΩNJ·ΩĺƛŽyˊ2Ŧ . 5bC ljdzƛŎ]fè LJN2ŘdzŎŝă ŎdzΩĺ·yŝ2dză LJl·ƛyˊ l·yŘ ōfl·ŘŘŝN KèLJŝNJl·ŎΩĺƛǿƛΩè óCNΩl·ă ŝΩ l·f¢Σ нлмoú¢ !ŘŘƛΩĺƛ2yl·ffèΣ ƛyΩNJl·ΩKŝŎl·f . 5bC ōf2Ŏ]l·Řŝ ōè ¢NJ]. -LƎH 2N ¢NJ] NŝŎŝLJΩ2N l·yΩĺ·Ǝ2yăΩΣ]нрнl·Σ ǿl·ă ăK2ǿyˊ Ω2 NŝŘdzŎŝ LJl·ƛyˊ l·yŘ ōfl·ŘŘŝN KèLJŝNJl·ŎΩĺƛǿƛΩè ƛyˊ NJl·Ωă ǿƛΩK ŎKN2yƛŎ ōfl·ŘŘŝN ƛyŦfl·YˋYl·Ωĺ2yˊ óCNΩl·ă ŝΩ l·f¢Σ нлмoú¢ l ŝNΩŝΣ ƛyǾŝăΩƛƎl·ΩŝŘ ΩKŝ Ŏ2yΩNJƛōdzΩĺ2yˊ2Ŧ . 5bC Ω2 ΩKŝ ŝYˋŝNΩƎŝyŎŝ 2Ŧ b5h dzăƛyƎ l· NJl·Ω Yˋ2Řŝfˊ2Ŧ {/L¢

Material and Methods

Animals

CŝYˋl·fŝ ² ƛăΩĺ·NJ NJl·Ωă ŦN2Yˊ /Kl·NfŝăwƛǾŝN óCNJl·yŎŝΩ ǿŝƛƎKƛyƎ нрл-нтр Ǝ ǿŝNΩŝ dzăŝŘ¢ !yƛYˋl·fă ǿŝNΩŝ]ŝLJΩ 2yˊмн K ŘJl·NJ]kfƛƎKΏ ŎèŎfŝΣ ƛyˊl· ΩŝYˋLJŝNJl·ΩdzNΩŝ Ŏ2yΩN2ffŝŘ ŝyǾƛNΩ2yYˋŝyΏ ǿƛΩK *ad libitum* l·ŎŎŝăă Ω2 Ŧ22Ř l·yŘ ǿl·ΩŝN¢ !ff ŝŦŦ2NΩΩă ǿŝNΩŝ Yˋl·Řŝ ƛyˊ 2NΩŘŝN Ω2 NŝŘdzŎŝ l·yƛYˋl·f ăΩNΩăă l·yŘ ădzŦŦŝNΩƛyƎ l·ă ǿŝff l·ă ΩKŝ ydzYˋŌŝN 2Ŧ l·yƛYˋl·fă dzăŝŘ¢ ¢Kŝ ŝΩĺKƛŎl·f ƎdzƛŘŝfΩƛySă Ŧ2NJ ƛyǾŝăΩƛƎl·ΩĺƛŽyˊ 2Ŧ ŝÈLJŝNΩYˋŝyΩĺ·f LJl·ƛyˊ ƛyˊ l·yƛYˋl·fă ó½ƛYˋYˋŝNJYˋl·yyΣ мфуoú l·yŘ ΩKŝ 9dzN2LJŝl·yˊ /2YˋYˋăăƛ2yˊ 5ƛNΩŝŎΩĺǾŝ 2Ŧ нн {ŝLJΩŝYˋŌŝN нлмл óнлмлксок9¦ú ǿŝNΩŝ Ŏl·NJŝŦdzffè Ŧ2ff2ǿŝŘ ƛyˊ l·ff LJN2ŎŝŘdzNΩŝă ƛyŎfdzŘŝŘ ƛyˊ ΩKƛă ăΩdzŘè¢

Chemicals and reagents

!ff ădzNƎŝNΩƛŝă ǿŝNΩŝ LJŝNŦ2NJYˋŝŘ dzyŘŝN ŘŝŝLJ l·yl·ŝăΩKŝăƛl· ƛyŘdzŎŝŘ ōè ƛyΩNJl·LJŝNΩΩ2yŝl·f ƛy?ŝŎΩĺƛ2yˊ2Ŧ l·YˋlÈΩdzNΩŝ 2Ŧ YˋŝŘŝΩĺ2YˋlŘƛyŝ ól¢нр YˋƎкYƎú l·yŘ]ŝΩl·Yˋƛyŝ ócл YˋƎкYƎΩΣ ŘlfdzΏŝŘ ƛyˊ ăΩŝNΩlfŝ ăl·flyŝ¢ C2N ŎèăΩ2YˋŝΩNΩŝă l·yŘ ΩŝNJYˋƛyl·f Kl·yŘflyƎΣ NJl·Ωă NŝŎŝΩǾŝŘ l·ădzōŎdzΏl·yŝ2dză ō2fdză 2Ŧ dzNΩŝΩKl·yŝ óм¢н ƎкYƎú l·ă l·yl·ŝăΩKŝΩƛŎ¢

¢Kŝ NŝŎ2Yˋōƛyl·yΏ LJN2Ωŝƛyˊ ¢N]. -LƎHΣ ǿKƛŎK ƛă l·yˊ ƛYˋYˋdzyˊ2Ǝfƒōdzfƛy-fƛ]ŝ Ř2Yl·ƛyˊ ΩKl·Ώ ōƛyŘă Ω2 . 5bC ǿΩK LJƛŎ2Yˋ2fl·NJ l·ŦŦƛyΏŝΣ ǿl·ă LJN2ŘdzŎŝŘ ƛyˊ K2dzăŝ l·yŘ ŘƛfdzΏŝŘ ƛyˊ нл Yˋa ¢NΩăă ōdzŦŦŝNJ LJl yфнΣ млл Yˋa bl·/f l·yŘ м£?ŝ ƎfèŎŝN2f ó. l·yŦƛŝfŘ ŜΩ l·f¢Σ нллмT bl·èf2NJ ŜΩ l·f¢Σ нллнú ¢ .5bC óbŝdzN2YˋƛŎăΣ ¦{!úΣ ăl·flyŝ l·yŘ ¢N]. -LƎHΣ ǿŝNΩŝ ŘŝfƛǾŝNΩŝŘ ƛyΩ2 ΏKŝ ădzōl·NJl·ŎKy2ƛŘ ăLJl·ŎŝΣ ØƛlˊŽăY2ΩƛŎ YˋƛyƛLJdzYˋLJă ó!fÎŝΩΣ ¦{!ú¢ {dzōăΏl·yŎŝă ǿŝNΩŝ ŘŝfƛǾŝNΩŝŘ Ŧ2NJ т óY2Řŝfˊ нллмú 2NJ ну Řl·ëă óY2Řŝfˊ нллпú¢

!yΩƛō2Řè l·Ǝl·ƛyăΏ .5bCΣ Yˋl·ŘŝΣ ƛyˊ NJl·ōōƛΩΣ ǿl·ă ō2dzƎKΏ ŦN2Yˊ aƛffƛLJ2NΩŝ óNΩŜŦY !.мттфΣ ¦{!ú¢ wl·ōōƛΏ l·yΩĺƛ-DN2ǿΩK !ăă2ŏƛl·ΩŝŘ tN2Ώŝƛyˊ по óD!t-поú ŎlˊYˋŝ ŦN2Yˊ !ōŎlˊY ó¦Yú¢ wl·ōōƛΏ l·yΩĺƛ-LJK2ăLJK2WbY ǿl·ă LJdzNΩŎKl·ăŝŘ ΩŝŘ /Ŝff {ƛƎyl·fƛyƎ ó¦{!ú¢ ¢KŝΣ l·yΩĺƛō2Řè l·Ǝl·ƛyăΏ /l·fŎƛΏ2yƛyˊ DŝyŜ-wŜfl·ΩŝŘ tŜLJΩƛŘŝS ó/Dwtú ǿl·ă NJl·ăŝŘ ƛyˊYˋ2dzăŝ l·yŘ ŎlˊYˋŝ ŦN2Yˊ !ōŎlˊYˋΣ ¦Y¢ ¢KŝΣ l·yΩĺƛ -ōŜΩĺ·-LLL ΩdzōdzfƛyˊYˋl·ŘŝΣ ƛyˊYˋ2dzăŝ ŎlˊYˋŝ ŦN2Yˊ tN2YˋŜƎl·Σ ¦{!¢ ¢KŝΣ ŦfdzN2N2ŎN2YˋŝΣ fl·ōŜŦfŜŘ l·yΩƛō2ŘƛŝădzăŜŘΣ !fŜÈl·ϰ-ŦfdzN2N pcy Ř2yˊ]Ŝè l·yΩƛ-NJl·ōōƛΏ l·yŘ !fŜÈl·ϰ -ŦfdzN2N пуу Ǝ2l·Ώ l·yΩƛ-YˋŽdzăŜΣ ŎlˊYˋŝ ŦN2Yˊ a2fŜŎdzfl·N tN2ōŜăσΣ ¦{!¢ ¢KŝΣ ōƛ2ΩƛyˊŎ2yˊdzƎl·ΩŜŘ ăǿƛyŝ l·yΩƛ-NJl·ōōƛΏ l·yŘ ΩKŝ !ØƛŘƛyˊ-.ƛ2Řƛyˊ /2YˋLJfŜÈ ó!./ú ±ŜŎăΏl·ăΩĺl·ƛyˊ 9fƛΩŝ]ƛΩΣ Ŏ2yˊdzƎl·ΩŜŘ ǿΩK K2NΩăŜNJl·ŘƛăK LJŝN2ÈƛŘl·ăŝ ólwtúΣ ǿŝNΩŝ l·Ôljdz NΩŝŘ NΩŜăLJŝŎΩƛǾŜfè Ŧ2Yˊ 5l·]2LJl·ΩĺΩă !кр ó5ŝyYˋl·NJ]ú l·yŘ ±ŝŎΏ2N [l·ō2NJl·Ώ2NΩƛŜă ó¦Yú¢ !ff l·yΩƛō2Řƛŝăƛ l·yŘ ΩKŝ !./ Ŏ2YˋLJfŜÈ ǿŝNΩŝ LJNΩŜLJl·NΩŝŘ ƛyˊ LJK2ăLJKl·ΩŝŘ ōdzŦŦŝNΩŝŘ ăl·fƛyŝ л¢м a ót.{ú Ŏ2yΩĺ·ƛyƛyƎ л¢?ŝ ¢NΩĺΩ2yˊ ··млл ót.{¢ú¢

C2NJ ŎŝΩff ŎdzfΏdzNΩŝΣ 5dzfōŝŎŎ2Ωă a2ŘƛŦƛŝŘ 9l·ƎfŝaŝŘƛdzYˋ Cмн ó5a9a-CмнúΣ ŦŝΩĺ·f ō2ǿƛyŝ ăŝNΩdzYˋ óC.{úΣ LJŝyƛŎƛffƛyƙăúNΩŜLJΏ2YˋèŎƛyˊ ótŝyƙ{úNΩŜLJΩú l·yŘ [-DfdzfΏl·YˋƛyŝŎl·YˋŝŦN2Yˊ LyǾƛΩN2ƎŜyΣ ¦{!¢ /2ffl·ƎŜyl·ăŝ ó¢ëLJŝ L±-{úΣ ō2ǿƛyŝ ăŝNΩdzYˋ l·fōdzYˋƛyˊ ó.{!ú LJ2fè-[-fèăƛyŝ l·yŘ fl·Yˋƛyƛyˊ ǿŝNΩŝ LJdzNΩŎKl·ăŝŘ ŦN2Yˊ {ƛƎYˋl·Σ 9dzN2LJŝ¢ .нтΣ l· ăŝNΩdzYˋ-ŦNΩŜŝ ădzLJLJfŜYˋŜyΏ Ŧ2N yŜdzNJl·f ŎŝffŎdzfΏdzNΩŜΣ Ŏl·YˋŝŦN2Yˊ DƛōŎ2Σ ¦{! ǿKŝΩŝl·ă yŜNΩǾŝ ƎN2ǿΏK Ŧl·ŎΏ2N н¢p{ óbDCú ŎlˊYˋŝ ŦN2Yˊ aƛffƛLJ2NΩŜΣ ¦{!¢

Spinal cord transection and cystometry

¢Kŝ Yˋ2Řŝfˊ 2Ŧ {/L ŎK2ăŜyˊŦ2N ΩKŝ LJNΩŝăŜyΏ ădzŘè ǿl·ă ŎKN2yƛŎ ălJƛyl·f Ŏ2NΩŘ ΩNJl·yăŜŎΩƛ2y¢ CŝYˋl·fŝ ² ƛăΩĺ·N NJl·Ωă óyΓc l·yƛYˋl·fă LJŝNΩ ŜÈLJŝNΩƛYˋŝyΏŝl·f ƎN2dzLJú dzyŘŝNΩǿŝyΏ ădzNΩƎƛŎl·f ƛYˋLJfl·yΏl·Ωƛ2yˊ 2Ŧ l· ălfƛŎ2yŝ Ŏl·ΩKŝΩŝNJ ó{C aŝŘƛŎl·fΣ IdzŘă2yΣ a!Σ ¦{!ú óYŝNΩN ŜΩ l·f¢Σ мфффT ¢K2YˋLJă2yˊ ŜΩ l·f¢Σ мфффT /NdzÎ ŜΩ l·f¢Σ нллрT /NdzÎ ŜΩ l·f¢Σ нллсT CNΩl·ă ŜΩ l·f¢Σ нлмoú Ŧ2ff2ǿŝŘ ōè Ŏ2YˋLJfŜΩŝ ălJƛyl·f Ŏ2NΩŘ ΩNJl·yăŜŎΩƛ2y¢ /l·ΩKŝΩŝNΩă ǿŝNΩŝ LJfl·ŎŝŘ ƛyΩ2 ΩKŝ fdzYˋŌl·N ădzōl·NJl·ŎKy2ƛŘ ălJl·ŎŝΣ l·Ώ ΩKŝ [pκ[c ălJƛyl·f Ŏ2NΩŘ fŝǾŜf¢ .NΩŜŦfèΣ l· fl·YˋƛyŝŎΏ2Yè ǿl·ă LJŝNΩŦ2NJYˋŝŘ ōŝΏǿŜŝyˊ¢ф l·yŘ ¢мл l·yŘ ΩKŝ YˋŝyƛyƎŜă ǿŝNΩŝ LJƛŜNΩŎŝŘ¢ ¢KŝΣ ŎlˊΩKŝΩŝNJ ǿl·ă ƛyăŜNΩΩŝŘ dzyΩŘŝNJ ΩKŝ ădzōl·NJl·ŎKy2ƛŘ YˋŜYˋōNJl·yŝ l·yŘ LJdzăKŜŘ dzyΏƛf ΩKŝ ΏƛLJ NΩŜl·ŎKŜŘ ΏKŝ [p-[c ălJƛyl·f Ŏ2NΩŘ ăŝƎYˋŝyΏ¢ ¢KŝΣ ălJƛyl·f Ŏ2NΩŘ ǿl·ă ΩKŝyˊ Ŏ2YˋLJfŜΩŝfè ăŜŎΩƛ2yŝŘ l·Ώ ¢ф fŝǾŝf l·yŘ ăΩŝNΩƛfŝ ƎŝfŦ2l·Y ǿl·ă LJfl·ŎŝŘ ōŝΏǿŜŝyˊ ΩKŝ NΩŝΩNJl·ŎΏŝŘ ŝyŘă 2Ŧ ΩKŝ Ŏ2NΩŘ¢ ¢KŝΣ 2ΩKŝNJ ŝyŘ 2Ŧ ΩKŝ ΩƛLJ 2Ŧ ΩKŝ Ŏl·ΩKŝΩŝNJ ǿl·ă Ŏ2yŷŝŎΩŝŘ Ώ2 l·yˊ 2ăY2ΩƛŎ YˋƛyƛLJdzYˋLJ ó Ŧ2N Ŏ2yΏƛyˊdz2dzăŘŝfǾŝNΩŝè 2Ŧ ălfƛyŝΣ ¢NJ]. -LƎH 2N .5bC - ăŝŜ ōŝf2ǿúΣ LJfl·ŎŝŘ ōŝΏǿŜŝyˊ ΩKŝ ăK2dzfŘŝNJ ōfl·ŘŝăŜ l·yŘ fŝŦΏ Ŧ2NJ м 2NJ п ǿŝŜ]ă¢ Lyˊ l·ƎN2dzLJ 2Ŧ {/L NJl·Ωă ΏKŝΣ Ŏl·ΏKŝΩŝNJ ǿl·ă fŝŦΏ ădzōŎdzΏl·yŝ2dzăfè l·yŘ ŜÈΩŝNyl·fƛÎŝŘ п ǿŝŜ]ă l·ŦΩŝNJ {/L Ŧ2NJ l·ŎdzΩŝ ŘNdzƎ ŘŝfƛǾŝNè¢

!ff l·yƛYˋl·fă ǿŝNΩŝ Ŏl·NJŝŦdzffè Yˋ2yƛΩ2NΩŝŘ l·yŘ NΩŝŎŝƛǾŝŘ l· Řl·λfè ƛyΩNJl·LJŝNΩΩ2yŝl·f ƛy?ŝŎΩĺƛ2yˊ 2Ŧ l·yΩĺƛōƛ2ΏƛŎ óŎƛLJN2ŦfŦ2È l·ŎƛyΣ м YˋƎк]ƎΩ Ŧ2NJ Ώǿ2 ǿŝŜ]ă l·ŦΩŝNJ ădzNΩŝƎŝNè¢ ¢2 l·Ǿ2ƛŘ dzNΩƛyl·NJè NΩŝΩŝyΏƛ2yˊ ōfl·ŘŘŝNΩă ǿŝNΩŝ Yˋl·ydzl·ffè ŜYˋLJΩƛŝŘ ōè l·ōŘ2Yˋƛyl·f Ŏ2YˋLJNΩŝăăƛ2yˊ ΏǿƛŎŝ ŜǾŝNè Řl·è¢ .ŜŎl·dzăŜ ΏKƛăLJN2ŎŝŘdzNΩŝ ƛă Yˋ2NΩŜ

difficult to perform in male rats, female animals were preferred to conduct the present study. In one group of SCI animals, the subcutaneous end of the catheter was externalized four weeks after surgery. Animals received sequential intrathecal injections of sterile saline or TrkB-Ig_2 (1µg, 10 µg and 20 µg in a volume of 25 µl) every 30 minutes while bladder reflex activity was being registered. Injection of TrkB-Ig_2 was followed by a flush of the same volume of saline to assure that all recombinant protein was injected into the subarachnoid space.

In three groups of SCI animals, the intrathecal catheter was connected to an osmotic mini-pump for continuous delivery of sterile saline, TrkB-Ig_2 (20 µg/day) or BDNF (1.5 µg/day) for seven days, according to previous studies (Frias et al., 2013). On the seventh day, bladder reflex activity was evaluated by cystometry. In three other groups of SCI rats, as above, the silicone catheter was connected to an osmotic mini-pump for continuous delivery of sterile saline, BDNF (0.7 µg/ day and 2.1 µg/day) for a period of 28 days. The smallest dose was chosen as potentially producing effects on bladder function without cutaneous pain (Frias et al., 2013) whereas the highest dose was based on the effects observed with 0.7 µg/ day.

For cystometry, bladders were exposed through a low abdominal midline incision and a 21-gauge needle was inserted into the bladder dome for saline infusion. Animals were left untouched for fifteen to thirty minutes to allow bladder stabilization. Body temperature was maintained at 36-37 ºC with a heating pad. The urethra remained unobstructed throughout the experiment so that infused saline could easily be expelled by bladder contractions. After bladder stabilization, saline infusion was initiated and bladder reflex activity was recorded for approximately 30 minutes. Saline was infused through the dome needle at a constant rate of 6 mL/h whilst bladder contractions were registered by a pressure transducer (WPI Instruments) connected to a computer.

At the end of the experiments, animals were perfused and the position of the catheter verified. The cystometrograms obtained were analysed. The frequency, peak pressure, area under the curve (AUC) and amplitude of bladder contractions were determined in the different phases of the experiment.

Perfusion and immunohistochemistry

After cystometry, animals were perfused through the ascending aorta with cold oxygenated calcium-free Tyrode's solution (0.12 M NaCl, 5.4 mM KCl, 1.6 mM, $MgCl_2 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $NaH_2PO_4.H_2O$, 5.5 mM glucose, 26.2 mM $NaHCO_3$), followed by 4 % paraformaldehyde. The dissection of the perfused nervous tissue allowed the confirmation of the position of the intrathecal catheter. Only animals in which the catheter was correctly placed were considered for further analysis. The spinal cord segments L5-L6 were collected, post-fixed for 4 h and cryoprotected for 24 h in 30 % sucrose with 0.1 % sodium azide in 0.1 M phosphate buffer. Transverse 40-µm sections of the collected spinal cord segments were cut in a freezing microtome and stored in cryoprotective solution at -20 ºC until all tissue was collected.

The expression of GAP-43 and phosphoJNK was tested using the ABC method. Briefly, sections were thoroughly washed in PBS. After inhibition of endogenous peroxidase activity and further washes in PBS and PBST, sections were incubated in 10 % normal swine serum in PBST for 2 h. Sections were then incubated for 48 h at 4 ºC with a specific antibody against GAP-43 (1:5000) or against phosphoJNK (1:500). Subsequently, sections were washed in PBST and incubated for 1 hour with polyclonal swine anti-rabbit biotin conjugated antibody (1:200). In order to visualize the immunoreactions, the ABC conjugated with peroxidase (1:200) method was used with 3, 3-diaminobenzidine-tetrahydrochloride as chromogen (DAB; 5 minutes in 0.05 M Tris buffer, pH 7.4 containing 0.05 % DAB and 0.003 % hydrogen peroxide). Sections were mounted on gelatine-coated slices and air-dried for 12 h, cleared in xylene, mounted with *Eukitt* mounting medium and cover-slipped.Alternate spinal sections were used to determine the colocalization between GAP-43 and CGRP. For this, sections were thoroughly washed in PBS and PBST, followed by a 2 hour-incubation in 10% normal horse serum in PBST. Sections were then incubated with anti-GAP-43 (1:5000) and anti-CGRP (1:8000) for 48 hours. Afterwards, sections were washed in PBST and incubated in fluorescently -labelled anti-mouse (1:1000) and anti-rabbit (1:1000) for 1 hour. Sections were then washed, mounted in Prolong Gold© mounting medium (Molecular Probes©, USA) and observed in a Z4 AxioImager Zeiss© microscope.

In vitro studies: Dorsal Root Ganglia (DRG) cell culture and immunocytochemistry

¢KŜ ŜÈLJŜNΩƛYŜyΩİ·f ƎN2dzLJä ŘŜăÕNΩƛōŜŘ İ·ō2ØŜ ØŜNΩŜ NΩŜLJfΩÖİ·ΩŜŘ Ŧ2N ÖÔŜff ÖdzfΩdz NΩŜ İ·yŘ ØŜăΩŜNΩY ōf2ΩΩƛyƎ óăŜŜ ōÔŜf2ØúΦ LY ΩKƛă ÖI·ăŜΣ ΩKŜ yΩzYYōŜN 2Ŧ İ·yƛYİ·fã LJŜN ŜÈLJŜNΩƛYŜyΩİ·f ƎN2dzLJ Ø İ·ä ΠΦ !ă ōŜŦ2NΩŜΣ İ·ff İ·yƛYİ·fã dzyŘŜNØŜyΩ ÖèăΩ2YŜΩNè dzyŘŜN dzNΩŜΩKİ·yŜ İ·yİ·ŜăΩKŜăƛI·Φ !ŦΩŜN ÖèăΩ2YŜΩNèΣ İ·yƛYİ·fã ØŜNΩŜ ŜdzΩKİ·yƛÎŜŘ İ·yŘ ΩKŜ [p Ω2 {м 5wD Ö2ffŜÖΩŜŘ İ·yŘ ƛYYŜŘƛI·ΩŜfè LJfİ·ÖŜŘ ƛy 5a9a-CмнΣ мΩ C.{Σ tŜyк{ΩNΩLJΦ 5wDä ØŜNΩŜ ΩKŜy ƛyÖdzōİ·ΩŜŘ ØƛΩK лΦмнΩ Ö2ffI·ƎŜyİ·ăŜ Ŧ2N н K İ·Ω отс/Φ !ŦΩŜN ΩKNΩŜŜ Øİ·ăKŜă ƛy 5a9aкCмн YŜŘƛdzYΣ 5wDä ySdzN2yã ØŜNΩŜ NΩŜăădzăLJŜyŘŜŘ İ·yŘ Řƛăă2ÖƛI·ΩŜŘ ƛy 5a9a-CмнΣ мΩ C.{ İ·yŘ ΩΩ tŜyк{ΩNΩLJ ōè NΩŜLJŜI·ΩŜŘ LJƛLJŜΩΩƛyƎΦ ¢KŜ NΩŜădztΩƛyƎ ÔŜff ădzăLJŜyãƛ2y Øİ·ă ÔŜyΩNΩŦdzƎŜŘ İ·Ω млллNLJY Ŧ2N мл YƛyΩKN2dzƎK İ·н Yf ÖdzăKƛ2y 2Ŧ мΩ .{! Ŧ2N NΩSY2Øİ·f 2Ŧ ÔŜftdzfI·N ŘŜōNΩăΦ ¢KŜ NΩŜădztΩƛyƎ LJŜffŜΩΣ Ö2yΩİ·ƛyƛyƎ ΩKŜ ySdzN2yãΣ Øİ·ă NΩŜăădzăLJŜyŘŜŘ ƛy 5a9a-Cмн YŜŘƛdzY Ö2yΩİ·ƛyƛyƎ ΩΩ tŜyк{ΩNΩLJΣ [-DfdzΩİ·YƛyŜ óнлл Y aύΣ .нт óнл ҡfkYfΩ İ·yŘ лΦлр ҡƎкYf bDC¢ ¢KŜ ÔŜff ă2fdzΩƛ2y2ōΩİ·ƛyŜŘ Ŧ2N2Y ŜI·ÖK İ·yƛYİ·f Øİ·ă LJfİ·ΩŜŘ 2yΩ2 LJ2fè-[-fèăƛyŜ óнл ҡƎк YfΩ İ·yŘ fİ·Yƛyƛy-Ö2İ·ΩŜŘ óp ҡƎкYfΩ Ö2ØŜNΩăfΩLJă İ·yŘ Y İ·ƛyΩİ·ƛySŘ İ·Ω отс/ ƛy İ· KdzYƛŘƛŦΛŜŘ ΩΣ /hн İ·ΩY2ăLJKŜNΩŜΦ

¢ØŜfØŜ K2dzNΩă fİ·ΩŜNΣ ΩKŜ LJfİ·ΩŜŘ ÔŜffă ØŜNΩŜ ŦÀÈŜŘ ØƛΩK ƛÖŜ-Ö2fŘΩΣ LJİ·NİŦ2NYİ·fŘŜKèŘŜ Ŧ2N ŦƛŦΩŜŜyY ƛyΩdzΩŜă İ·Ω N22Y ΩŜYLJŜNİ·ΩdzNΩŜΦ ¢KŜ ÔŜffă ØŜNΩŜ Ø İ·ăKŜŘ ΩKNΩŜŜ ΩƛYŜă ƛy t.{Σ Ŧ2ff2ØŜŘ ōè İ· p-YƛyΩzΩŜ ƛyÖdzōİ·ΩƛY ƛy t.{ лΦΩ ¢NΩƛ2yΦ !ŦΩŜNØİ·NŘăΣ ÔŜffă ØŜNΩŜ İ·Ǝİ·ƛy Ø İ·ăKŜŘ ƛy t.{ İ·yŘ ƛyÖdzōİ·ΩŜŘ Ŧ2N p YƛyΩzΩŜă ƛy İ·ă2fdzΩƛ2y 2Ŧ лΦΩ 2Ŧ ă2ŘƛdzY ō2N2KιŘNΩŘŜΦ ¢Kƛă Ø İ·ă Ŧ2ff2ØŜŘ ōè o Ø İ·ăKŜă ƛy t.{ İ·yŘ м K2dzN ƛyÖdzōİ·ΩƛY İ·Ω N22Y ΩŜYLJŜNİ·ΩdzNΩŜ ƛy ōf2Ö]ƛyƎ ă2fdzΩƛ2y óΩ 2Ŧ C.{ ƛy лΦΩ t.{-¢ØŜŜyнлύΦ !ŦΩŜN ōf2Ö]ƛyƎΣ ÔŜffă ØŜNΩŜ ƛyÖdzōİ·ΩŜŘ ƛy İ·yΩƛ-ōŜΩİ·-LLL ΩdzōdzfΩy óмYнллл ƛy ōf2Ö]ƛyƎ ōdzŦŦŜNΩύ Ŧ2N м K2dzN İ·Ω N22Y ΩŜYLJŜNİ·ΩdzNΩŜΦ ¢KŜ ÔŜffă ØŜNΩŜ ΩKŜy Ø İ·ăKŜŘ o ΩƛYŜă ØƛΩK t.{ İ·yŘ ƛyÖdzōİ·ΩŜŘ ØƛΩK ΩKŜ ăŜÖ2yŘİ·Nè İ·yΩƛō2ŘèΣ !fŜÈİ·ϰ-ŦfdzN2N пyy Ǝ2İ·Ω İ·yΩƛ-Y2dzăŜ óмYмллл ƛy ōf2Ö]ƛyƎ ōdzŦŦŜNΩύΦ ¢Kƛă ăΩŜLJ Ø İ·ă Ŧ2ff2ØŜŘ ōè Ø İ·ăKŜă ƛy t.{ İ·yŘ Y2dzyΩƛyƎ ƛy ±ŜÖΩİ·ăKƛŜfŘ YŜŘƛdzY ó±ŜÖΩ2N [İ·ō2Nİ·Ω2NΩŜăΣ ¦YΩΣ İ·ŦΩŜN ØKƛÔK ăfΩŘŜă ØŜNΩŜ ăŜİ·fŜΠΦ wŜLJNΩŜăŜyΩİ·ΩΩŜ ƛYİ·ƎŜă ØŜNΩŜ Ø2ffŜÖΩŜŘ ƛy İ·y !Èƛ2ăÖ2LJŜ пл YƛÔN2ăÖ2LJŜ ØƛΩK ΩKŜ !Èƛ2±ăƛ2y пΦc ă2ŦΩØİ·NΩŜ ó/İ·Nf ½ŜƛăăŦú Ŧ2N İ·yİ·fèăƛă 2Ŧ ySdzNΩƛΩŜ ōNİ·yÔKƛyƎΣ ΩKŜ Ω2Ωİ·f ySdzNΩƛΩŜ fŜyƎΩK İ·yŘ ΩKŜ İ·NŜİ· 2Ŧ ΩKŜ ă2YİΦ

Quantification and Statistics

/èăΩ2YŜΩN2ƎNİ·Yă ØŜNΩŜ İ·yİ·fèăŜŘ dzăƛyƎ ΩKŜ [İ·ō{ÔNΩƛōŜ ă2ŦΩØİ·NΩŜ ó±ŜNΩăƛ2y нΦопфллΣ L ² 2NÈ ăèăΩŜYă LyÖΦΩΦ ¢KŜ ŦNŜljdzŜyÔè İ·yŘ İ·YLJfΩΩdzŘŜ 2Ŧ ōfİ·ŘŘŜN Ø2yΩNİ·ÖΩƛ2yãΣ LJŜİ·] LJNΩŜăădzNΩŜ İ·yŘ İ·NŜİ· dzYŘŜN ΩKŜ ÖdzNØŜ ó!¦/ύ ØŜNΩŜ İ·yİ·fèăŜŘ ōè dzăƛyƎ YNdză]İ·f-² İ·ffăă 2ySŜ-Øİ·è NΩŜLJŜİ·ΩŜŘ Y Ŝİ·ădzNΩŜăΦ

Results

Bladder function and spinal BDNF expression after SCI

¢KŜ ŜŦŦŜÖΩă 2Ŧ {/L 2y ōfİ·ŘŘŜN NΩŜŦfŜÈ İ·ÖΩƛØƛΩè ØŜNΩŜ ƛyØŜăΩƛƎİ·ΩŜŘ м İ·yŘ п ØŜŜ]ă İ·ŦΩŜN ăLJƛyİ·f Ø2NΩŘ fŜăƛ2yΦ ¢KŜ LJİ·ΩΩŜNΩY 2Ŧ ōfİ·ŘŘŜN NΩŜŦfŜÈ İ·ÖΩƛØƛΩè ƛy ăLJƛyİ·f ƛyΩİ·ÖΩ İ·yƛYİ·fãΣ ƛy ØKİ·Ω Ö2yÖŜNΩyã ΩKŜ Øİ·fdzŜă 2Ŧ ŦNŜljdzŜyÖèΣ LJŜİ·] LJNΩŜăădzNΩŜΣ İ·YLJfΩΩdzŘŜ İ·yŘ ! ¦ / 2Ŧ ōfİ·ŘŘŜN NΩŜŦfŜÈ Ö2yΩNİ·ÖΩƛ2yã ƛă ăK2ØyY ƛy CƛƎΦ м! İ·yŘ CƛƎăΦ p!-p5 óΦİ·ØfŜ мΩΦ hyŜ ØŜŜ] İ·ŦΩŜN {/L Øfİ·ŘŘŜN NΩŜŦfŜÈ Ö2yΩNİ·ÖΩƛ2yã ØŜNΩŜ LJNΩİ·ÖΩƛÖİ·ffè İ·ō2fΩKŜŘ ƛy İ·ÖÖ2NΩŘİ·yÖŜ ØƛΩK ΩKŜ 2ÖÖdzNΩNΩŜyÖŜ 2Ŧ ăLJƛyİ·f ăK2Ö] óCƛƎΦ HŌ İ·yŘ CƛƎăΦ p!-p5T ¢İ·ØfŜ мΩΦ C2dzN ØŜŜ]ă İ·ŦΩŜN {/L b5h Ø İ·ă ŜØƛŘŜyΩ ƛy İ·ff {/L İ·yƛYİ·fã óCƛƎΦ м/ύΣ ØƛΩK ăƛƎyΛŦƛÖİ·yΩ ƛyÖNΩŜİ·ăŜ ƛyΩΩKŜ Øİ·fdzŜă 2Ŧ ŦNΩŜljdzŜyÖèΣ LJŜİ·] LJNΩŜăădzNΩŜΣ İ·YLJfΩΩdzŘŜ İ·yŘ ! ¦ / óCƛƎăΦ p!-p5T ¢İ·ØfŜ мΩΦ

9ÈLJNΩŜăăƛ2y 2Ŧ .5bC Ø İ·ă Ŧ2dzyŘ ΩKN2dzƎK2dzΩ ΩKŜ ƎNİ·è Yİ·ΩΩŜN 2Ŧ ΩKŜ ăLJƛyİ·f Ö2NΩŘΣ ΩKŜ ăΩN2yƎŜăΩ ƛYYdzy2NΩŜİ·ÖΩƛ2y ØŜƛyƎ ō2ăŜNΩØŜŘ ƛy ΩKŜ Ř2NΩăİ·f K2NΩyã óCƛƎΦ м5Σ м9Σ мCΩΦ ¢KŜ ăLJŜÖƛŦΛÖƛΩè 2Ŧ ΩKŜ İ·yΩƛō2Řè Kİ·Ř İ·fNΩŜİ·Řè ōŜŜy ΩŜăΩŜŘ ŜfăŜØKŜNΩŜ óCNΩƛİ·ă ŜΩ İ·fΦΣ нлмоύ İ·yŘ y2 ƛYYdzy2NΩŜİ·ÖΩƛ2y Øİ·ă 2ōăŜNΩØŜŘ ØKŜy ăŜÖΩƛ2yã ØŜNΩŜ ƛyÖdzōİ·ΩŜŘ ƛy ΩKŜ İ·ōăŜyÖŜ 2Ŧ LJNΩƛYİ·NΩè İ·yΩƛō2Řè óŘİ·Ωİ· y2Ω ăK2ØyΩΦ !yİ·fèăƛă 2Ŧ ΩKŜ ƛyΩŜyãƛΩè 2Ŧ ΩKŜ ƛYYdzy2ăΩİ·ƛyƛyƎ ăK2ØŜŘ İ· ΩƛYŜ-ŘŜLJŜyŘŜyΩ ƛyÖNΩŜİ·ăŜ ƛy ăLJƛyİ·f fŜØŜfă 2Ŧ .5bC İ·ŦΩŜN {/L ΩKŜ ăΩN2yƎŜăΩ ƛyΩŜyãƛΩè ōŜƛyƎ 2ōăŜNΩØŜŘ п ØŜŜ]ă İ·ŦΩŜN {/L óLJғлΦллм ØŜNΩădză ăLJƛyİ·f ƛyΩİ·ÖΩΦ

BDNF sequestration in rats with established NDO

5dzNΩƛyƎ ÖèăΩ2YŜΩNèΣ п ØŜŜ]ă {/L NΩİ·Ωă NΩŜÖŜƛØŜŘ ƛyÖNΩŜİ·ăƛyƎ Ř2ăŜă 2Ŧ ¢N]. -LƎH ŜØŜNè ол YƛyΩzΩŜă ƛy İ·ÖdzYdzfI·ΩƛØŜ Y İ·yŶŜNΦ ² Ŝ Ŧ2dzyŘ ΩKİ·Ω İ· Ř2ăŜ-ŘŜLJŜyŘŜyΩ .5bC ăŜljdzŜăΩNΩİ·Ωƛ2y ƛYLJNΩ2ØŜŘ ōfİ·ŘŘŜN ŦdzyÖΩƛ2y óCƛƎăΦ н!-н9T ¢İ·ōfŜ мΩΦ !Ω Ř2ăŜă 2Ŧ мл ҡƎ 2Ŧ ¢N]. -LƎH ōfİ·ŘŘŜN NΩŜŦfŜÈ Ö2yΩNİ·ÖΩƛ2yã ØŜNΩŜ Ōİ·ăƛÔİ·ffè İ·ō2fΩăKŜŘΦ ¢KŜăŜ NΩŜădztΩă ădzƎƎŜăΩŜŘ ΩKİ·Ω .5bC ƛă ƛyØ2fØŜŘ ƛy b5h İ·NΩăƛyƎ İ·ŦΩŜN {/LΦ

Effect of BDNF sequestration during spinal shock

.ŜÔİ·dzăŜ ΩKŜ ƛyÖNΩŜİ·ăŜ 2Ŧ ăLJƛyİ·f .5bC İ·yŘ ΩKŜ ŜŦŦŜÖΩă 2Ŧ .5bC ăŜljdzŜăΩNΩİ·Ωƛ2y ƛy п ØŜŜ]ă {/L NΩİ·Ωă ădzƎƎŜăΩŜŘ ΩKİ·Ω .5bC Ø İ·ă fƛy]ŜŘ Ω2 ΩKŜ ŜYŜNƎŜyÖŜ 2Ŧ b5hΣ {/L NΩİ·Ωă ØŜNΩŜ ădzōOYΩΩŜŘ Ω2 .5bC ăŜljdzŜăΩNΩİ·Ωƛ2y ŘdzNΩƛyƎ ΩKŜ ŦNΩăΩ ØŜŜ] İ·ŦΩŜN ăLJƛyİ·f Ö2NΩŘ ΩNΩİ·yãŜÖΩƛ2y ōè ÖKN2yÖ ŘŜfΩØŜNΩè 2Ŧ ¢N]. -LƎH.Φ .5bC ăÖİ·ØŜyƎƛyƎ LJNΩ2Y2ΩŜŘ İ·y Ŝİ·NΩfΩŜNΩ İ·LJLJŜİ·NΩİ·yÖŜ 2Ŧ b5h óCƛƎΦ o!ΩΣ İ·ă ăK2ØyY ōè İ· ăƛƎyΛŦƛÖİ·yΩ ƛyÖNΩŜİ·ăŜ

2Ŧ Ŧ₪ŜIjdzŜyÖè I·yŘ I·Y˝LJfƛŭdzŘŜ 2Ŧ ōfI·ŘŘŜ₪ Ö2yŭ₪I·Öŭƛ2yãΣ LJŜI·ʼl LJ₪ŜăădZ₪Ŝ I·yŘ ! ¦ / ƛy˝ м ǿŜŜl {/L I·yƛY˝I·fã ú₪ŜI·ŭŜŘ ǿƛúK ¢₪l . -LƎH ƛy˝ Ö2Y˝LJI·₪Ăă2y˝ ǿƛúK y2y-ú₪ŜI·ŭŜŘ м ǿŜŜl {/L I·yƛY˝I·fã óLJғл¢лpT CƛƎă p!-p9T ¢I·ōfŜ мú¢¢KŜ fI·ŭúŜ₪ Ǝ₪2dzLJ 2Ŧ I·yƛY˝I·fã ŜÈKƛōƛű 2ōØƛ2dză ăƛƎyã 2Ŧ ăLJƛyl·f ăK2Ölʼ¢ LY˝Ydzy2ăűI·ƛyƛyƎ 2Ŧ ăLJƛyl·f ăŜÖŭƛ2yã 2ōűI·ƛyŜŘ Ŧ₪2Y˝ {/L I·yƛY˝I·fã ădzōY˝ŭűŜŘ ű2 .5bC ăŜIjdzŜăú₪I·ŭƛ2y˝ Ö2yŦ₪₪Y˝ŜŘ ŭKŜ ădzÖÖŜää 2Ŧ ŭKŜ ú₪ŜI·ű˝YŜyű ōè I· Y˝I·₪ʼlŜŘ ŘŜÖ₪ŜI·äŜ ƛy˝ ăLJƛyl·f .5bC ăűI·ƛyƛyƎ óCƛƎ¢ o.ú¢ ¢KŜăŜ ŘI·ŭI· ădzƎƎŜăű ŭKI·ű .5bC ŘdzNƛyƎ ŜI·₪fè LJŜNƛ2Ř ŭKI·ű Ŧ2ff2ǿă ăLJƛyl·f Ö2₪Ř ú₪I·yãŜÖŭƛ2y˝LJ₪ŜØŜyú ₪I·úKŜ₪ ŭKI·y˝Ö2yú₪ƛdzúŜ ű2 b5h¢

Effect on bladder function of chronic intrathecal administration of BDNF to SCI rats

{/L ₪I·ûă ₪ŜÖŜƛØŜŘ Ö2yűƛydz2dzătè ƛyú₪I·ŭKŜÖI·f .5bC Ŧ2₪ м 2₪ п ǿŜŜlă¢ .5bC I·ŘY˝ƛyƛăú₪I·ŭƛ2y˝ ǿI·ă ƛyƛűƛI·ŭŜŘ ƛY˝Y˝ŜŘƛI·ŭŜfè I·ŦűŜ₪ {/L¢ LyÚ₪I·ŭKŜÖI·f I·ŘY˝ƛyƛăú₪I·ŭƛ2y˝ 2Ŧ .5bC óм¢p ĸƎĸŘI·èú Ŧ2₪ м ǿŜŜl LJ₪2ŘdzÖŜŘ y2 ÖKI·yŜă ƛy˝ŭKŜ LJI·ŭűŜ₪y˝ 2Ŧ ōfI·ŘŘŜ₪ ₪ŜŦfŜÈ I·ÖűƛØƛúèΣ ǿKƛÖK ǿI·ă ƛyŘƛăűƛyƎdzƛăKI·ōfŜ Ŧ₪2Y˝ ŭKI·ű 2ōăŜ₪ØŜŘ ƛy˝y2y-ú₪ŜI·ŭŜŘ м ǿŜŜl {/L I·yƛY˝I·fã óCƛƎă¢ п!Σ p!-p5T ¢I·ōfŜ мú¢ LyÚ₪I·ŭKŜÖI·f I·ŘY˝ƛyƛăú₪I·ŭƛ2y˝ 2Ŧ .5bC óл¢т ĸƎĸŘI·èú ű2 {/L ₪I·ûă ŘdzN˝ƛyƎ п ǿŜŜlă KI·ŘΣ ƛy˝Ö2yú₪I·ăúΣ I· Y˝I·₪ʼlŜŘ ŜŦŦŜÖű 2y˝ōfI·ŘŘŜ₪ Ŧdzyÿűƛ2y¢ Ly˝Ö2Y˝LJI·₪ă2y˝ ǿƛűK y2y-

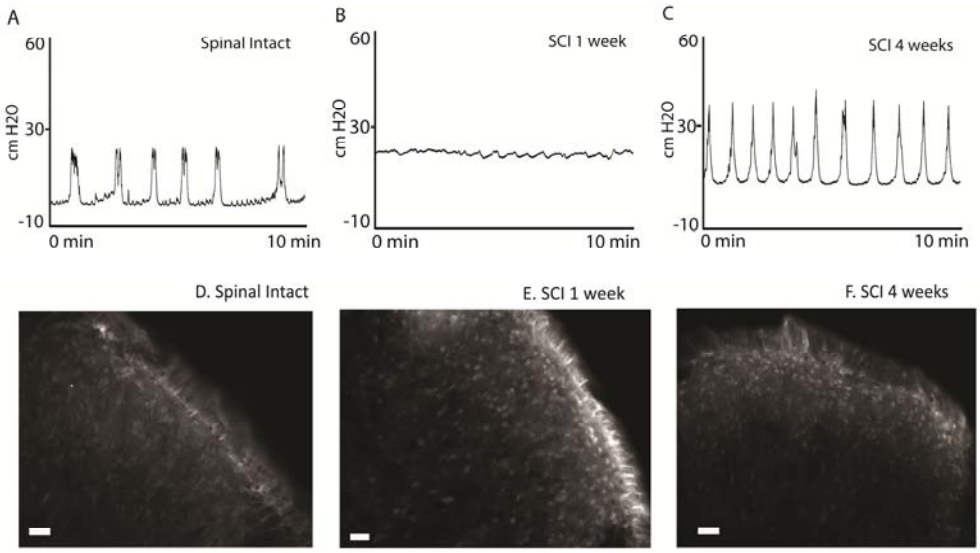

Figure 1. (A-C) Representative cystometrograms of spinal intact and spinal cord injured (SCi) animals. ó!ú Ly˝ăLJƛyl·f ƛyűI·Öű I·yƛY˝I·fãΣ úKŜ LJI·úűŜ₪y˝2Ŧ ōfI·ŘŘŜ₪ ŧdzyÖŭƛ2y˝ ǿI·ă y2₪Y˝I·f ődzű ăƎyŦƛÖI·yűfè I·fűŜ₪ŜŘ I·ú 2yŜ ǿŜŜl I·ŦűŜ₪ ăLJƛyl·f Ô2₪Ř ƛydz₪è ó.úΣ ǿKŜy˝ ōfI·ŘŘŜ₪ ₪ŜŦfŜÈ I·ÖűƛØúê ǿI·ă Ô2Y˝LJfŜűŜfê I·ō2fűăKŜŘ¢ ó/ú C2dz₪ ǿŜŜlă {/L I·yƛY˝I·fã LJ₪ŜăŜyűŜŘ ōfI·ŘŘŜ₪ KèLJŜ₪I·ÖűƛØúê¢ **(D-F) Photomicrographs depicting BDNF expression in the L5-L6 spinal cord segment of spinal intact, one week and four weeks SCI animals. Scale bar = 20 µm.** Ly˝ŭKŜ ăLJƛyl·f Ö2₪ŘΣ .5bC ŜÈLJ₪ŜăăƛƎ2y˝ǿI·ă Ŧ2dzyŘ ƛyÖ₪ŜI·ăŜŘ ƛy˝ŭKŜ Ř2₪ăI·f K2₪yã 2Ŧ ŭKŜ ăLJƛyl·f Ô2₪Ř¢ Lú ăƎyŦƛÖI·yűfè ƛyÖ₪ŜI·ăŜŘ 2ØŜ₪ ûƛY˝Ŝ I·ŦűŜ₪ {/L ƛy˝fI·Y˝ƛyl·Ŝ L I·yŘ LL óFLJғл¢лp I·yŘ FFFLJғл¢ллм ØŜ₪ădzã ăLJƛyl·f ƛyűI·ÖűΣ ₪ŜăLJŜÖűƛØŜfè¢

Table 1. ±I·fdzŜă 2Ŧ Ŧ₪ŜIjdzŜyÖèΣ LJŜI·ʼl LJ₪ŜăădZ₪Ŝ I·yŘ I·Y˝LJfƛŭdzŘŜ 2Ŧ ōfI·ŘŘŜ₪ ₪ŜŦfŜÈ Ô2yŭ₪I·Öűƛ2yã 2Ŧ ăLJƛyl·f ƛyűI·Öű I·yƛY˝I·fãΣ y2y-ú₪ŜI·ŭŜŘ {/L ₪I·ûă I·yŘ {/L ₪I·ûă ₪ŜÔŜƛØƛyƎ ¢₪ʼl . -LƎH 2₪ .5bC¢ 5dz₪ƛyƎ ŘƛăŜI·ăŜ LJ₪2Ǝ₪Ŝăăƛ2yΣ úKŜ₪Ŝ ǿI·ă I·y˝ƛyÖ₪ŜI·ăŜ 2Ŧ I·ff LJI·₪I·Y˝ŜúŜ₪ă¢ .5bC ăŜIjdzŜăú₪I·ŭƛ2y˝ŘdzN˝ƛyƎ ăLJƛyl·f ăK2Ölʼ ƛyŘdzÔŜŘ ŜI·₪fè b5hΣ I·ă I·ff LJI·₪I·Y˝ŜúŜ₪ă ǿŜ₪Ŝ KƛƎKŜ₪ úKI·y˝{/L I·yƛY˝I·fã I·ú ŭKŜ ăI·Y˝Ŝ ŭƛY˝Ŝ LJ2Iyú¢ !ŘY˝ƛyƛăú₪I·ŭƛ2y˝2Ŧ úKŜ f2ǿŜăŭ Ř2ăŜ 2Ŧ .5bC ǿI·ă 2yfè ŜŦŦŜÖűƛØŜ ƛŦ LJ₪2f2yƎŜŘ Ŧ2₪ п ǿŜŜlăΣ ǿKŜ₪Ŝl·ă ƛyÖ₪ŜI·ăŜyƎ ŭKŜ I·Y˝2dzyú 2Ŧ b¢ ŘƛŘ y2ú ƛY˝LJ₪2ØŜ ōfI·ŘŘŜ₪ ŧdzyÖŭƛ2y¢

FLJғл¢лp ØŜ₪ădzã !Σ FFFLJғл¢ллм ØŜ₪ădzã !Σ ILJғл¢лp ØŜ₪ădzã /Σ III LJғл¢ллм ØŜ₪ădzã /Σ P LJғл¢лp ØŜ₪ădzã .Σ 9 LJғл¢лp ØŜ₪ădzã C

	Spinal intact (group A)	SCI 1 week (group B)	SCI 4 weeks (group C)	SCI 4 wks + 1, 10, 20 µg TrkB-Ig2 (group D; values refer to 20 µg)	SCI + BDNF sequestration during spinal shock (group E)	SCI + BDNF 0.7µg/day for 4 weeks (group F)	SCI + 2.1 µg/ day for 4 weeks (group G)
Frequency	л¢рбл¢м	л¢лпбл¢л^{F}	м¢нбл¢о^{F}	л¢обл¢н^{F}	м¢нбл¢р^{FI}	л¢тобл¢м^{I}	м¢нбл¢пF^{9}
Peak pressure	нм¢рбн¢ф	нф¢лбт¢н	пн¢убп¢н^{FFF}	ом¢лбр¢с	от¢нбмм¢т^{F}	нс¢убн¢л^{I}	по¢тбн¢фF^{9}
Amplitude	му¢тбо¢р	н¢сбл¢т^{FFF}	ну¢тбо¢у^{FFF}	с¢мб о¢ф^{F}	мл¢тбн¢ф^{FFF}	му¢нбо¢о^{III}	нп¢лбс¢п^{9}

{/L I·yƛY I·fã I·Ω п ØŜŜ]ăΣ ØŜ ₮2dzyŘ ΩKI·Ω лΦт кƎкŘI·è 2₮ . 5bC ŘŜŎNŜI·ăŜŘ ΩKŜ ₣NΩŜIjdzŜyŎèΣ I·YLJfƛΩdzŘŜΣ LJŜI·] LJNΩŜăădzNΩŜ I·yŘ ! ¦ / 2₮ ōfI·ŘŘŜN Õ2yΩNI·ÕΩÚƛ2yã óCƛƎăΦ п . Σ p!-p5T ¢I·ōfŜ мΩΦ /KN2yƛÕ . 5bC I·ŘYƛyƛăΩNI·Úƛ2y I·Ω KƛƎKŜN Ř2ăŜă ŘdzNΩƛyƎ п ØŜŜ]ă óнΦм кƎкŘI·èÚ ŘƛŘ y2Ω LJNΩ2ŘdzŎŜ I·yê ōSyŜ₮ƛŎƛI·f ŜŦŦŜŎÚ I·ă ōfI·ŘŘŜN ₮dzyŎΩÚƛ2y Ø I·ă ăƛYƛfI·N ΩÚ2 y2y-ÚNΩŜI·ΩŜŘ п ØŜŜ]ă {/L I·yƛY I·fã óCƛƎăΦ п/Σ p!-p5T ¢I·ōfŜ мΩΦ /2NNΩŜŎΩ I·ŘYƛyƛăΩNI·Úƛ2y 2₮ ΩKŜ b¢ Ø I·ă Õ2y₮ΛNΩYŜŘ ōè ŜØI·fdzI·ΩƛyƎ . 5bC ƛYY dzy2-NΩŜI·ŎΩƛØƛΩêΣ ØKƛŎK Ø I·ă ăƛƎyƛ₮ΛŎI·yΩfè ƛyŎNΩŜI·ăŜŘ ƛy Õ2Y-LJI·NJăă2y Øƛ Ω K y2y-ÚNΩŜI·ΩŜŘ {/L NJI·Ωă I·Ω ΩKŜ ăI·YŜ ΩƛYŜ LJ2ƛyΩ óLJ₣ΦлpúΦ ¢KŜăŜ NΩŜădzfΩă ădzƎƎŜăΩŜŘ ΩKI·Ω LJNΩ2-f2y3ŜŘ . 5bC I·ŘYƛyƛăΩNI·Úƛ2y YI·è LJNΩŜØŜyΩ ΩKŜ I·LJ-LJŜI·NJI·yÕŜ 2₮ b5hΦ ¢Kλă LJNΩ2ΩŜÕΩƛØŜ ŜŦŦŜŎΩ ƛă K2ØŜØŜN f2ăΩ ƛ₮ KΛƎK Ř2ăŜă 2₮ . 5bC I·NΩŜ dzăŜŘΦ

Ly I·ff Õl·ăŜăΣ ăLJƛyΩ·f . 5bC ŜÈLJNΩŜăăƛ2y Ø I·ă I·ăăŜăăŜŘ Ω2 Õ2y₮ΛNY ₮dzff . 5bC ŘŜ₮ØŜNΩè I·yŘ ØŜ 2ōăŜNØŜŘ ăΩNΩ2yƎŜNJ ƛYY dzy2NΩŜI·ŎΩƛØƛΩÚè ƛy ăLJƛyΩ·f ăŜÕΩƛ2yã ₣N2Y ÚNΩŜI·ΩŜŘ I·yƛY I·fã ƛy Õ2YLJI·NJă2y ØƛΩK y2y-ÚNΩŜI·ΩŜŘ {/L I·Ω ΩKŜ ăI·YŜ ΩƛYŜ LJ2ƛyΩ óCƛƎăΦ п9Σ пCΣ пDΩΦ

GAP-43 expression at the spinal cord

¢KŜ I·LJLJŜI·NJI·yÕŜ 2₮ b5h I·₮ΩŜN {/L KI·ă ōŜŜy fƛy]ŜŘ Ω2 ălJN2dzΩƛyƎ I·yŘ ăèyΩ·LJΩƛÕ NŜ2N∃I·yƛÎ I·Ωƛ2y 2₮ ōfI·ŘŘŜN âŜyã2N è I·₮₮ŜNŜyΩă¢ ¢KŜăŜ ŜØŜyΩă Õl·y ōŜ Y2yƛΩ2NŜŘ ōè I·yΩ·fèăƛyƎ D!t-по ŜÈLJNΩŜăăƛ2yΣ I·YI·NJ]ŜN 2₮ I·È2yΩ·f ălJN2dzΩƛyƎ ó±ƛÎÎI·NŘΣ мфффú¢ D!t-по ƛYYdzy2NΩŜI·ÕΩÚƛ2y Ø I·ă ØŜNè Y2ŘŜăΩ ƛy ălJƛyΩ·f ăŜÕΩƛ2yã ₣N2Y ălJƛyΩ·f ƛyΩI·ÕΩ NΩI·Ωă óCƛƎ¢ c!Ω hy ΩKŜ Õ2yΩNJI·NèΣ D!t-по ŜÈLJNΩŜăăƛ2y ăƛƎyƛ₮ƛÕI·yΩfè ƛyÕNΩŜI·ăŜŘ ƛy I· ΩƛYŜ-ŘŜLJŜyŘŜyΩ YI·yŷŜN I·₮ΩŜN {/L óLJ₣ΦллмΩŜNădză ălJƛyΩ·f ƛyΩI·ÕΩT CƛƎ¢ c.Σ c/Σ cCΩ¢ Ly ΩKŜăŜ I·yƛYI·fã ƛYYYdzy2fI·ōŜftƛyƎ ØI·ă ₮2dzyŘ ōƛfI·ΩŜNI·ffè ƛy ΩKŜ ădzLJŜN₮ƛÕƛI·f fI·èŜNă 2₮ ΩKŜ Ř2NăI·f K2NyΦ D!t-по ØI·ă I·fØI·èă Õ2-f2ÕI·fÎŜŘ ØƛΩK /Dwt óCƛƎ¢ cDΣ cIΣ cLÚΦ

/2yΩƛydz2dză . 5bC ăŜIjdzŜăΩNJI·Ωƛ2y ŘdzNΩƛyƎ ΩKŜ ălJƛyΩ·f ăK2Õ] NŜădzfΩŜŘ ƛy ƛyΩŜyãŜ D!t-по ŜÈLJNΩŜăăƛ2yΣ ƛy Õ2YLJI·NΩă2y ØƛΩK ălJƛyΩ·f ƛyΩI·ÕΩ NJI·Ωă óLJ₣лplp ØŜNădzăălJƛyΩ·f ƛyΩI·ÕΩ T CƛƎ¢ c5Σ cCΩ¢ !ă ƛy ΩKŜ 2ΩKŜN ŜÈLJŜNΩY ŜyΩI·f ƎN2dzLJăΣ ălJN2dzΩƛyƎ I·È2yã ØŜNŜ /Dwt-LJ2ăƛΩƛØŜ óCƛƎΦ cWΩ¢

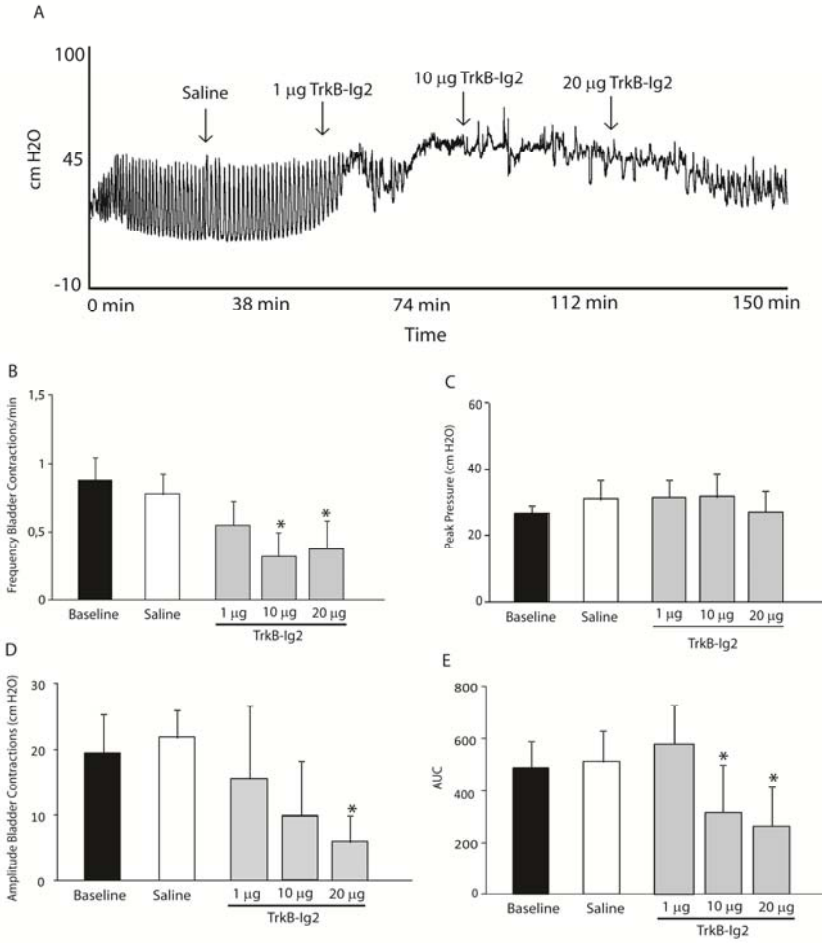

Figure 2. (A) Representative cystometogram of 4 weeks SCI animals treated with acute intrathecal injection of saline and TrkB-Ig$_2$. LyΩNI·ΩKŜÕI·f I·ŘYƛyƛăΩNI·Ωƛ2y 2₮ ăl·ΩyŜ ŘƛŘ y2ΩΩ I·fΩŜN ōfI·ŘŘŜN ₮dzyÕΩƛ2y ØKŜy Õ2YLJI·NΩŜŘ Ω2 Õl·ăŜΩyŜΦ Ly Õ2yΩNI·ăΩΣ ƛyΩNI·ΩKŜÕI·f ƛy2ŜÕΩƛ2y 2₮ ¢N] . -L∃$_H$ Ř2ăŜ-ŘŜLJŜyŘŜyΩfè I·Õ2ΩăKŜŘ ōfI·ŘŘŜN KèLJŜNI·ÕΩÚØΩÚè**Φ (B-E) Graph of bars depicting the mean frequency (B), peak pressure (C) and amplitude (D) of bladder contractions and area under the curve (AUC; E) of four weeks SCI animals treated with saline, 1 µg, 10 µg and 20 µg of TrkB-Ig$_2$.** ¢KŜ ₣NŜIjdzŜyŎèΣ I·YLJfƛ-ΩdzŘŜ 2₮ ōfI·ŘŘŜN Ô2yΩNI·ÕΩƛ2yã I·yŘ ! ¦ / ălƎyƛ₮ƛÕI·yΩfè ŘŜÕNŜI·ăŜŘ I·ΩŜN ƛyΩNI·ΩKŜÕI·f ƛy2ŜÕΩƛ2y 2₮ мл кƎ I·yŘ нл кƎ 2₮ ¢N] . -L∃$_H$ óF LJ₣лplp ØŜNădzăōI·ăŜΩyŜ ØI·fdzŜăΩΦ b2 ÔKI·yƎŜă ØŜNŜ ₮2dzyŘ ƛy LJŜI·] LJNŜăădzNŜΦ

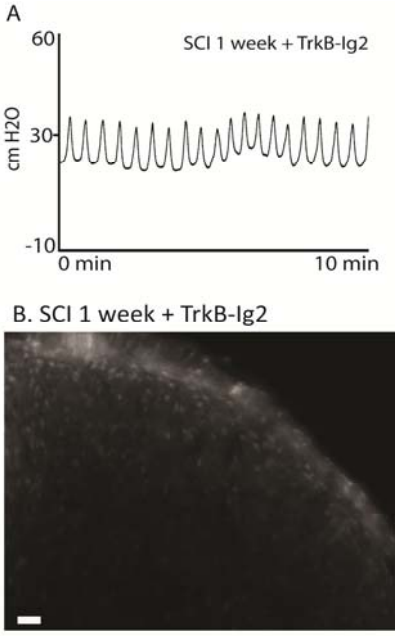

Figure 3. (A) Representative cystometrogram of 1 week SCI animals treated with TrkB-Ig$_2$. /KN2yƗÕ ƛyΨN·ΨKŜÕl·f . 5bC ãŜljdzŜãΨN·Ψƛ2yˇ ǿƛΨK ¢N] . -L3$_{\mu}$Σ ƛyΨƛl·ΨŜŘƛl·ΨŜfê l·ŦΨŜN ãLJƛyl·f Õ2NΨŘ ƛyDdzNΨè l·yŘ fl·ãΨƛyƎ Ŧ2N м ǿŜŜ]Σ NŜãdztΨŜŘ ƛyˇõfl·ŘŘŜN KèLJŜN·ÕΨƛØΨêΦ **(B) Photomicrograph showing BDNF expression in the L5-L6 spinal cord segment of 1 week SCI animals treated with TrkB-Ig$_2$. Scale bar = 20 µm.** LyΨN·ΨKŜÕl·f ãŜljdzŜãΨN·Ψƛ2yˇ 2Ŧ . 5bC Õl·dzãŜŘ l· Yl·N]ŜŘ ŘŜÕNŜl·ãŜ ƛyˇ . 5bC ŜÈLJNŜãƛ2yˇ ƛyˇΨKŜ Ř2NƛÍ·f K2NyˇƛyˇÕ2YLJl·NƛãyˇØƛΨK yDy-ΨNŜl·ΨŜŘ м ǿŜŜ] { /L Nl·Ψã óãŜŜ ŦƛƎΦ мT ғLJғлΦлр ØŜNƛdzã yDy-ΨNŜl·ΨŜŘ { /L Nl·Ψã l·Ψ ΨKŜ ãl·YŜ ΨƛYŜ LJ2ƛyΨúΦ

/2yΨƛyDz2dzã . 5bC ãŜljdzŜãΨN·Ψƛ2yˇ ŘdzNΨƛyƎ ΨKŜ ãLJƛyl·f ãK2Õ] NŜãdztΨŜŘ ƛyˇƛyΨŜyãŜ D ! t-no ŜÈLJNŜããƛ2yΣ ƛyˇ Õ2YLJl·Nƛãyˇ ǿƛΨK ãLJƛyl·f ƛyΨÍ·ÕΨŤ CƛƎãΦ c5Σ cCΨΦ ! ã ƛyˇ ΨKŜ 2ΨKŜN ŜÈLJŜNΨƛYŜyΨÍ·f ƎN2dzLJãΣ ãLJN2dzΨƛyƎ l·È2yã ǿŜNŜ /Dwt-LJ2ãΨƛΨØŜ óCƛƎΦ cWΨΦ

9ÈLJNŜããƛ2yˇ 2Ŧ D ! t-no ǿl·ã l·fã2 l·yl·fêãŜŘ l·ŦΨŜN п ǿŜŜ]ã 2Ŧ ƛyΨNl·ΨKŜÕl·f . 5bC l·ŘYˇƛyƛãΨNl·Ψƛ2yˇ ólΦтκƎk Řl·èΨ l·ŦΨŜN {/LΦ ! ã ƛyˇ ΨKŜ 2ΨKŜN ŜÈLJŜNΨƛYˇŜyΨÍ·f ƎN2dzLJãΣ D ! t-no ǿl·ã 2õãŜNØŜŘ ƛyˇ ΨKŜ ãdzLJŜNŦƛÕƛl·f fl·èŜNã 2Ŧ ΨKŜ Õ2NŘΦ D ! t-no ƛYˇYˇdzyˇ2NŜl·ÕΨƛ2yˇ ǿl·ã yˇ2Ψ l·ã ƛyΨŜyãŜ l·ã ΨKl·Ψ 2õãŜNØŜŘ ƛyˇ yˇ2y-ΨNŜl·ΨŜŘ п ǿŜŜ]ã {/L NΨl·Ψã óCƛƎΦ c9Σ cDΨΦ . 5bC ΨNŜl·ΨYˇŜyΨ ŘƛŘ yˇ2Ψ ŘŜÕNŜl·ãŜ D ! t-no ƛYˇYˇdzyˇ2NŜl·ÕΨƛØΨêˇ Ř2ǿyˇΨ2 fŜØŜfã 2Ŧ ãLJƛyl·f ƛyΨÍ·ÕΨ NΨl·Ψã óLJғлΦллмΨΦ

In vitro assessment of intrinsic growth ability

! ã ƛyÕNΨŜl·ãŜŘ fŜØŜfã 2Ŧ D ! t-no ƛyŘƛÕl·ΨŜ ƛyÕNΨŜl·ãŜŘ ƎN2ǿΨK l·õƛfƛΨêΣ 5wD ǿŜNŜ Õ2ffŜÕΨŜŘ ŦN2Yˇ l·ff ŜÈLJŜNΨƛYˇŜyΨÍ·f ƎN2dzLJã l·yŘ ySdzN2yã ÕdzfΨdz NΨŜŘ Ŧ2N мн K2dzNΨä Ψ2 ƛyØŜãΨƛƎl·ΨŜ ΨKŜNΨ ƛyΨNΨƛyΨãƛÕ ƎN2ǿΨK l·õƛfΨêΦ ! ff ySdzN2yã l·ŘKŜNŜŘ Ψ2 ΨKŜ ÕŜff ãdzõãΨNl·ΨŜ l·yŘ ŜYˇƛΨΨŜŘ f2yƎ ySdzNΨΨŜã óCƛƎãΦ т ! - т9ΨΦ bŜdzNΨΨŜ õNl·yÕKƛyƎ ǿl·ã ΨKŜ LJl·Nl·YˇŜΨŜN ΨKl·Ψ ãK2ǿŜŘ YˇN2NŜ Øl·NΨƛl·Ψƛ2yˇ l·ÕN2ãã ΨKŜ ŘƛŦŦŜNŜySyΨÍ·f ŜÈLJŜNΨƛYˇŜyΨÍ·f ƎN2dzLJãΦ . Nl·yÕKƛyƎ ǿl·ã ãƛƎyƛƛÕl·yΨfê l·fΨŜNŜŘ õè {/LΣ l·ã м ǿŜŜ] l·ŦΨŜN ƛyΦdzNΨè ΨKŜ YˇŜl·yˇ yΦzYˇõŜN 2Ŧ õNl·yÕKŜã LJŜN ÕŜff ǿl·ã ãƛƎyƛƛÕl·yΨfê f2ǿŜN ΨKl·yˇƛyˇÕŜffã 2õΨl·ƛySŘ ŦN2Yˇ ãLJƛyl·f ƛyΨÍ·ÕΨ Nl·Ψã óLJғлΦллм ØŜNãdzã ãLJƛyl·f ƛyΨÍ·ÕΨŤ CƛƎãΦ т . T y ! T ¢l·õfŜ нΨΦ Lyˇ Õ2yΨNl·ãΨΣ õNl·yÕKƛyƎ ǿl·ã YˇdzÕK Yˇ2NŜ LJN2YˇƛySyΨ ƛyˇ 5wD ySdzN2yãΣ LJl·NΨÍÕdzfl·NΨfê ƛyˇ ΨKŜ ØƛÕƛyΨΨê 2Ŧ ΨKŜ ã2Yl·Σ 2õΨl·ƛySŘ ŦN2Yˇ Nl·Ψã ǿƛΨK b5h óп ǿŜŜ]ã l·ŦΨŜN {/LT CƛƎãΦ т/Σ y . ΨΦ

Lyˇ {/L l·yΛYˇl·fã ãdzõYˇΨΨŜŘ Ψ2 . 5bC ãŜljdzŜãΨNl·Ψƛ2yˇ ŘdzNΨƛyƎ ΨKŜ ŦΛNãΨ ǿŜŜ] l·ŦΨŜN ΨNl·yãŜÕΨƛ2yΣ ySdzNΨΨŜ õNl·yÕKƛyƎ ǿl·ã ãƛƎyƛƛÕl·yΨfê ƛyÕNΨŜl·ãŜŘ óLJғлΦллм ØŜNãdzã ãLJƛyl·f ƛyΨÍ·ÕΨ l·yŘ {/L м ǿŜŜ]T CƛƎãΦ т5Σ y ! T ¢l·õfŜ нΨΦ

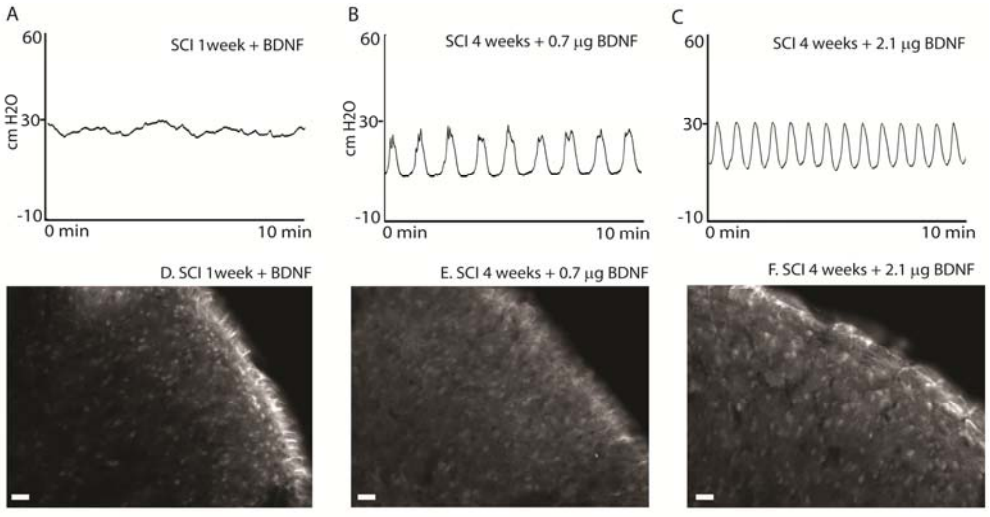

Figure 4. (A-C) Representative cystometrograms of 1 week and 4 weeks SCI animals treated with chronic BDNF (1.5 µg/day for 7 days, 0.7 µg/day and 2.1µg/day, for 28 days). Scale bar=20 µm. ó ! Ψ LyΨN·ΨKŜÕl·f l·ŘYˇƛyƛãΨNl·Ψƛ2yˇ 2Ŧ . 5bC Ŧ2N м ǿŜŜ]Σ l·ãƛ2 ƛyΨƛl·ΨŜŘ ƛYˇYˇŜŘƛl·ΨŜfê l·ŦΨŜN Õ2NŘ ƛyDdzNΨΣ ŘƛŘ yΨƛ LJN2ŘdzÕŜ ãƛƎyƛƛÕl·yΨ ÕKl·yƎŜã ƛyˇ õfl·ŘŘŜN ŦdzyÕΨƛ2yˇ ǿKŜyˇ Õ2YLJl·NŜŘ Ψ2 м ǿŜŜ] { /L l·yΛYˇl·fã° ó . Ψ /KN2yƗÕ ƛyΨNl·ΨKŜÕl·f l·ŘYˇƛyƛãΨNl·Ψƛ2yˇ 2Ŧ ΨKŜ fƛǿŜãΨ ŘƛŜ 2Ŧ . 5bC Ψ2 {/L NΨl·Ψã Ŧ2N п ǿŜŜ]ãΣ ǿKƛÕK õŜƎl·yˇƛYˇYˇŜŘƛl·ΨŜfê l·ŦΨŜN fŜãƛ2yˇ 2Ŧ ΨKŜ Õ2NŘΣ NŜãdztΨŜŘ ƛyˇ l·yˇƛYˇLJNƛ2ØŜYˇŜyΨ 2Ŧ õfl·ŘŘŜN ŦdzyÕΨƛ2yˇƛyˇ Õ2YˇLJl·Nƛãyˇ ǿƛΨK yDy-ΨNŜl·ΨŜŘ п ǿŜŜ]ã {/L NΨl·Ψã ó/Ψ ¢KŜ KƛƎKŜãΨ Ř2ãŜ 2Ŧ . 5bC ŘƛŘ yDΨ ƛyŘdzÕŜ ãƛƎyƛƛÕl·yΨ ƛYˇLJNƛ2ØŜYˇŜyΨã ƛyˇõfl·ŘŘŜN l·ÕΨƛØΨêΦ **(D-F) Photomicrographs showing BDNF expression in the L5-L6 spinal cord segment of 1 week and 4 weeks SCI animals treated with chronic BDNF (1.5 µg/day for 7 days, 0.7 µg/day and 2.1µg/day, for 28 days). Scale bar=20 µm.** . 5bC ŜÈLJNŜããƛ2yˇNŜYˇl·ƛySŘ ǿl·ã ŠfŜØl·ΨŜŘ ƛyˇΨKŜ Ř2NƛÍ·f K2NyˇƛΨ ΨKŜ ãLJƛyl·f ƛyˇ l·ff ŜÈLJŜNΨƛYˇŜyΨÍ·f ƎN2dzLJãΦ LyΨN·ΨKŜÕl·f l·ŘYˇƛyƛãΨNl·Ψƛ2yˇ 2Ŧ ΨKƛã b¢ ãƛƎyƛƛÕl·yΨfê ƛyÕNΨŜl·ãŜŘ . 5bC ŜÈLJNŜããƛ2yˇ l·Ψ ΨKŜ ãLJƛyl·f Ř2NƛÍ·f K2NyˇƛyΨ óғLJғлΦлр ØŜNãdzã yDy-ΨNŜl·ΨŜŘ { /L NΨl·Ψã l·Ψ ΨKŜ ãl·YŜ ΨƛYŜ LJ2ƛyΨúΦ

hűKŜN LJI·NI·Y˝ŜűŜNƛã fƛ]Ŝ ySdzNΩÚŜ fŜyƎűK I·yŘ I·NΩŜI· 2Ŧ ÚKŜ ă2YI· ăK2ǿŜŘ fŜăă ØI·NΩƛI·Úƛ2y˝ I·ÕNΩ2ăă ŜÈLJŜNΩƛY˝ŜyÚI·f ƎNΩ2dzLJãΦ bŜØŜNΩÚKŜfŜăăΣ ÚKŜN˝Ŝ ǿI·ã I· ăƛƎyƛŦƛÕI·yÚ NΩŜŘdzÕÚƛ2y˝ƛy˝ÚKŜ ySdzNΩÚŜ fŜyƎűK 2Ŧ 5wD ÕŜffã Õ2ffŜÕÚŜŘ M ǿŜŜ]I·ŦÚŜN {/L óLJ⨍ΦlpØŜNΩädzã ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fãúΦ

/KN2yƛÕ I·ŘY˝ƛyƛăÚNΩI·Úƛ2y˝ 2Ŧ .5bC I·fã2 ƛyÕNΩŜI·ăŜŘ ySdzNΩÚŜ ōNΩI·yÕKƛyƎΦ ¢N˝ŜI·ÚY˝Sy˝Ú ŘƛŘ y2ű I·ŦŦŜÕÚ ÚKŜ fŜyƎűK 2Ŧ ySdzNΩÚŜ ōdzÚ ōNΩI·yÕKƛyƎ ǿI·ã ŜØƛŘŜy˝Ú I·yŘ ătƛƎKÚfè Y2NΩŜ LJNΩ2Y˝ƛySy˝Ú ÚKI·y˝ ƛy˝ ÕŜffã 2ōÚI·ƛySŘ FN2Y˝ y2y˝ÚNΩŜI·ÚŜŘ n ǿŜŜ]ã {/L NΩI·ÚãΣ LJI·NÚƛÕdzfI·NΩfè ƛy˝ NΩŜƎƛ2yã f2ÕI·ÚŜŘ Y2NΩŜ ÚKI·y˝млл ƙY˝ FN2Y˝ ÚKŜ ă2YI· óCƛƎΦ т9Σ y.T ¢I·õfŜ нúΦ

Mechanisms of axonal sprouting: involvement of JNK

¢2 ƛyØŜăÚƛƎI·ÚŜ ÚKŜ ƛyÚNΩI·ÕŜffdzfI·N˝ Y˝ŜÕKI·yƛăYă 2Ŧ I·È2yI·f ƎN2ǿÚK˝Σ ǿŜ I·ăăŜăăŜŘ ÚKŜ fŜØŜfã 2Ŧ WbY I·ÕÚƛØI·Úƛ2y˝ƛy˝ ÚKŜ [c ăLJƛyI·f Õ2NΩŘ ăŜƎY˝Ŝyű¢ [ƛÚűfŜ LJK2ăLJK2WbY ƛY˝Y˝dzy˝2NΩŜI·ÕÚƛ2y˝ ǿI·ã Ŧ2dzyŘ ƛy˝ăŜÕ-Úƛ2yã FN2Y˝ ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fã óCƛƎăΦ ф!Σ фCΣ фDúΦ Ly˝ Õ2yÚNΩI·ăÚ˝ WbY I·ÕÚƛØI·Úƛ2y˝ 2ÕÕdzNΩNΩŜŘ ÚKN2dzƎK2dzű ÚKŜ Ř2NΩăI·f K2N˝y˝ƛy˝ {/L I·yƛYI·fã óCƛƎăΦ ф.-фDúΣ LJI·NÚƛÕdzfI·NΩfè ƛy˝ fI·Y˝ƛyI·Ŝ L I·yŘ LLΦ ¢N˝ŜI·ű-Y˝Syű ǿƛÚK .5bC ăÕI·ǿŜyƎŜN ŘdzNΩƛyƎ 2yŜ ǿŜŜ] ÕI·dzăŜŘ I· YI·N˝]ŜŘ ƛyÕNΩŜI·ăŜŘ 2Ŧ LJK2LJK2WbY ŜÈ-LJNΩŜăăƛ2yΦ I2ǿŜǿŜN˝Σ ÕKN2yƛÕ ÚNΩŜI·ÚY˝Syű ǿƛÚK .5bC ŘdzNΩƛyƎ n ǿŜŜ]ă ōN2dzƎKű WbY I·ÕÚƛØI·Úƛ2y˝ű2 ăLJƛyI·f ƛyÚI·Õű fŜØŜfs.

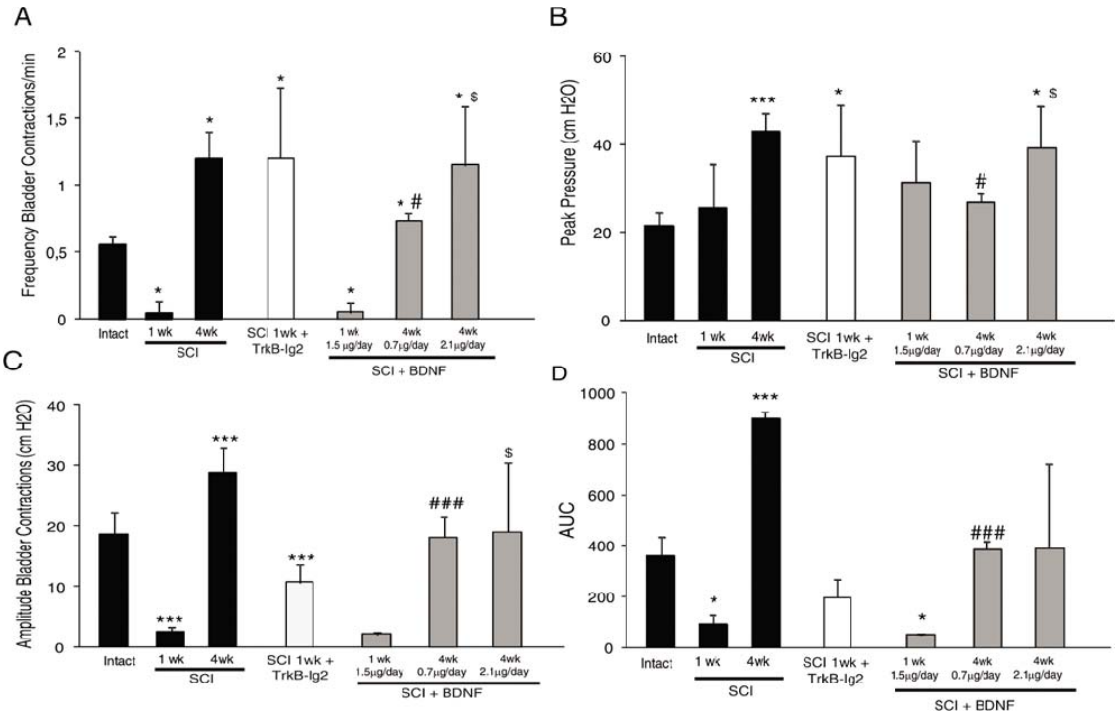

Figure 5. (A-D) Histograms showing the mean frequency, peak pressure and amplitude of bladder contractions and area under the curve (AUC) of spinal intact. óAú ¢KŜ FNŜIjdzŜyÕè 2Ŧ õfI·ŘŘŜN NŜŦfŜÈ Õ2yÚNΩI·ÕÚƛ2yã ǿI·ã ăƛƎyƛŦƛÕI·yűfè ŘŜÕNΩŜI·ăŜŘ M ǿŜŜ] I·ŦűŜN {/L ōdzÚ dzLJNΩŜƎdzfI·ÚŜŘ n ǿŜŜ]ă I·ŦűŜN Õ2NΩŘ ƛy˝dzNΩè óFLJ⨍ΦlpØŜNΩädzã ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fãúΦ hyŜ ǿŜŜ] {/L I·yƛYI·fã ƛyÚNΩI·ÚKŜÕI·ffè ÚNΩŜI·ÚŜŘ ǿƛÚK ÕKN2yƛÕ ¢N˝ . -LƎH LJNΩŜăŜyÚŜŘ I· ăƛƎyƛŦƛÕI·yÚ ƛyÕNΩŜI·ăŜ ƛy˝ õfI·ŘŘŜN NŜŦfŜÈ I·ÕÚƛØƛÚè ǿKŜy˝Õ2Y˝LJI·NΩŜŘ Ú2 ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fã I·yŘ ǿƛÚK y2y˝-ÚNΩŜI·ÚŜŘ I·yƛYI·fã I·Ú ÚKŜ ăI·YŜ ÚƛYŜ LJ2ƛyÚ óFLJ⨍ΦlpрÚΦ LyÚNΩI·ÚKŜÕI·f I·ŘY˝ƛyƛ-ăÚNΩI·Úƛ2y˝ 2Ŧ .5bC Ŧ2N˝ M ǿŜŜ] ŘƛŘ y2Ú I·ŦŦŜÕÚ õfI·ŘŘŜN Õ2yÚNΩI·ÕÚƛ2yã /KN2yƛÕ I·ŘY˝ƛyƛăÚNΩI·Úƛ2y˝ Ŧ2N˝ n ǿŜŜ] 2Ŧ .5bC NŜŘdzÕŜŘ dzNΩyI·NΩè FNŜIjdzŜyÕè ƛy˝Õ2Y-LJI·NΩă2y˝ ǿƛÚK y2y˝-ÚNΩŜI·ÚŜŘ {/L NΩI·Úã I·Ú ÚKŜ ăI·YŜ ÚƛYŜ LJ2ƛyÚ óILJ⨍ΦlpрÚΣ õdzÚ ÚKŜNΩŜ ƛã I·y˝ƛyÕNΩŜI·ăŜ ƛy˝ÚKŜ dzNΩyI·NΩè FNŜIjdzŜyÕè ǿKŜy˝Õ2YLJI·NΩŜŘ Ú2 ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fã óFLJ⨍ΦlpрÚΦ !ŘY˝ƛyƛăÚNΩI·Úƛ2y˝ 2Ŧ нΦм ƙƎkŘI·è Ŧ2N˝ n ǿŜŜ] ŘƛŘ y2Ú I·ŦűŜN dzNΩyI·NΩè FNŜIjdzŜyÕè óFLJ⨍ΦlpØŜNΩädzã ăLJƛyI·f ƛyÚI·Õű I·yŘ ÞLJ⨍ΦlpØŜNΩädzã nǿŜŜ] {/L ÚNŜI·ÚŜŘ ǿƛÚK лΦтƙƎkŘI·è 2Ŧ .5bCΦÚΦ

óBÚ C2dzN˝ ǿŜŜ] {/L I·yƛYI·fã I·yŘ 2yŜ ǿŜŜ] {/L I·yƛYI·fã ÚNŜI·ÚŜŘ ǿƛÚK ¢N˝ . -LƎH LJNΩŜăŜyÚŜŘ I·y˝ ŜfŜØI·Úƛ2y˝ 2Ŧ LJŜI·] LJNΩŜăădzNΩŜ ǿKŜy˝Õ2YLJI·NΩŜŘ Ú2 ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fã óFLJ⨍ΦlpрÚΦ .5bC I·ŘY˝ƛyƛăÚNΩI·Úƛ2y˝Ŧ2N˝ M ǿŜŜ] ŘƛŘ y2Ú I·ŦŦŜÕÚ LJŜI·] LJNΩŜăădzNΩŜ õdzÚ n ǿŜŜ] ÚNΩŜI·ÚY˝Syű ǿƛÚK f2ǿŜăÚ Ř2ăŜ 2Ŧ .5bC LJN2ŘdzÕŜŘ I· ăƛƎyƛŦƛÕI·yÚ NΩŘdzÕÚƛ2y˝ƛy˝Õ2YLJI·NΩă2y˝ ǿƛÚK {/L I·Ú ÚKŜ ăI·YŜ ÚƛYŜ LJ2ƛyÚ óILJ⨍ΦlpрÚΦ ØŜNΩädzã n ǿŜŜ] {/L NΩI·Úăú C2dzN˝ ǿŜŜ] {/L I·yƛYI·fã ÚNŜI·ÚŜŘ ǿƛÚK ÚKŜ KƛƎKŜăÚ Ř2ăŜ 2Ŧ .5bC LJNΩŜăŜyÚŜŘ I· ăƛƎyƛŦƛÕI·yÚ ƛyÕNΩŜI·ăŜ 2Ŧ ÚKŜ LJŜI·] LJNΩŜăădzNΩŜ ǿKŜy˝Õ2YLJI·NΩŜŘ Ú2 ăLJƛyI·f ƛyÚI·Õű óFLJ⨍ΦlpрÚΦ ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fã I·yŘ nǿŜŜ] {/L ÚNŜI·ÚŜŘ ǿƛÚK f2ǿŜăÚ Ř2ăŜ 2Ŧ .5bCΦÚΦ

óCÚ ! Y˝LJfÚúdzŘŜ 2Ŧ õfI·ŘŘŜN Õ2yÚNΩI·ÕÚƛ2yã ǿI·ã ăƛƎyƛŦƛÕI·yűfè NΩŘdzÕŜŘ M ǿŜŜ] I·ŦűŜN fŜăƛ2y˝ óFFFLJ⨍ΦlпмΩŜNΩädzã ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fãú I·yŘ I·y˝ƛyÕNΩŜI·ăŜ Ŧ2dzN˝ ǿŜŜ] {/L ōdzÚ dzLJNΩŜƎdzfI·ÚŜŘ n ǿŜŜ] ÚNΩŜI·ÚY˝Syű ǿƛÚK f2ǿŜăÚ Ř2ăŜ 2Ŧ .5bC LJN2ŘdzÕŜŘ I· ăƛƎyƛŦƛÕI·yÚ NΩŘdzÕÚƛ2y˝ƛy˝Õ2YLJI·NΩă2y˝ ǿƛÚK {/L I·Ú ÚKŜ ăI·YŜ ÚƛYŜ LJ2ƛyÚ óILJ⨍ΦlпмΩŜNΩädzã ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fã I·yŘ I·y˝ƛyÕNΩŜI·ăŜ Ŧ2dzN˝ ǿŜŜ] {/L I·yƛYI·fãúΦ .5bC ăŜIjdzŜăÚNΩI·Úƛ2y˝ ǿI·ã I·ÕÕ2YLJI·yƛŜŘ õè I· NΩŜŘdzÕÚƛ2y˝ 2Ŧ I·Y˝LJfÚúdzŘŜ 2Ŧ õfI·ŘŘŜN Õ2yÚNΩI·ÕÚƛ2yã ǿKŜNΩŜI·ă ÚKŜ LJN2f2yŜŘ I·ŘY˝ƛyƛăÚNΩI·Úƛ2y˝ ǿI·ã I·ÕÕ2YLJI·yƛŜŘ õè I· ăƛƎKÚ NΩŘdzÕÚƛ2y˝ ǿKŜy˝Õ2YLJI·NΩŜŘ ǿƛÚK n ǿŜŜ] {/L NΩI·Úã óIIILJ⨍ΦlпмΩŜNΩädzã Ŧ2dzN˝ ǿŜŜ] {/L I·yƛYI·fãúΦ

óDú ¢KŜ ! ¦ / ǿI·ã ǿŜNΩè ăY˝I·ff M ǿŜŜ] I·ŦűŜN fŜăƛ2y˝ õdzÚ ăƛƎyƛŦƛÕI·yűfè ƎNΩŜI·ÚŜNΩ ƛy˝ n ǿŜŜ] {/L I·yƛYI·fã óFLJ⨍ΦlpрÚΦ ǿŜNΩädzã ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fãúΦ b2 ÕKI·yƎŜă ǿŜNΩŜ Ŧ2dzyŘ ƛy˝M ǿŜŜ] {/L I·yƛYI·fã ÚNŜI·ÚŜŘ ǿƛÚK ¢N˝ . -LƎH ǿKŜy˝Õ2YLJI·NΩŜŘ Ú2 ăLJƛyI·f ƛyÚI·Õű I·yƛYI·fãΦ hyŜ ǿŜŜ] ÚNΩŜI·ÚY˝Syű ǿƛÚK лΦт ƙƎkŘI·è .5bC ŘƛŘ y2Ú I·ŦŦŜÕÚ ! ! / ƛy˝Õ2YLJI·NΩă2y˝ ǿƛÚK {/L I·yƛYI·fã I·Ú ÚKŜ ăI·YŜ ÚƛYŜ LJ2ƛyÚ óFLJ⨍ΦlpрÚΦ ǿŜNΩädzã ăLJƛyI·f ƛyÚI·Õű ōdzÚ n ǿŜŜ] ÚNΩŜI·ÚY˝Syű ǿƛÚK ÚKŜ ăI·YŜ Ř2ăŜ NΩŜŘdzÕŜ ! ¦ / ƛy˝Õ2YLJI·NΩă2y˝ ǿƛÚK y2y˝-ÚNΩŜI·ÚŜŘ I·yƛYI·fã I·Ú ÚKŜ ăI·YŜ ÚƛYŜ LJ2ƛyÚ óILJ⨍ΦlpрÚΦ ǿŜNΩädzã Ŧ2dzN˝ ǿŜŜ] {/L I·yƛYI·fãúΦ b2 ÕKI·yƎŜă ǿŜNΩŜ Ŧ2dzyŘ ǿƛÚK ÚKŜ KƛƎKŜăÚ Ř2ăŜ 2Ŧ .5bCΦ

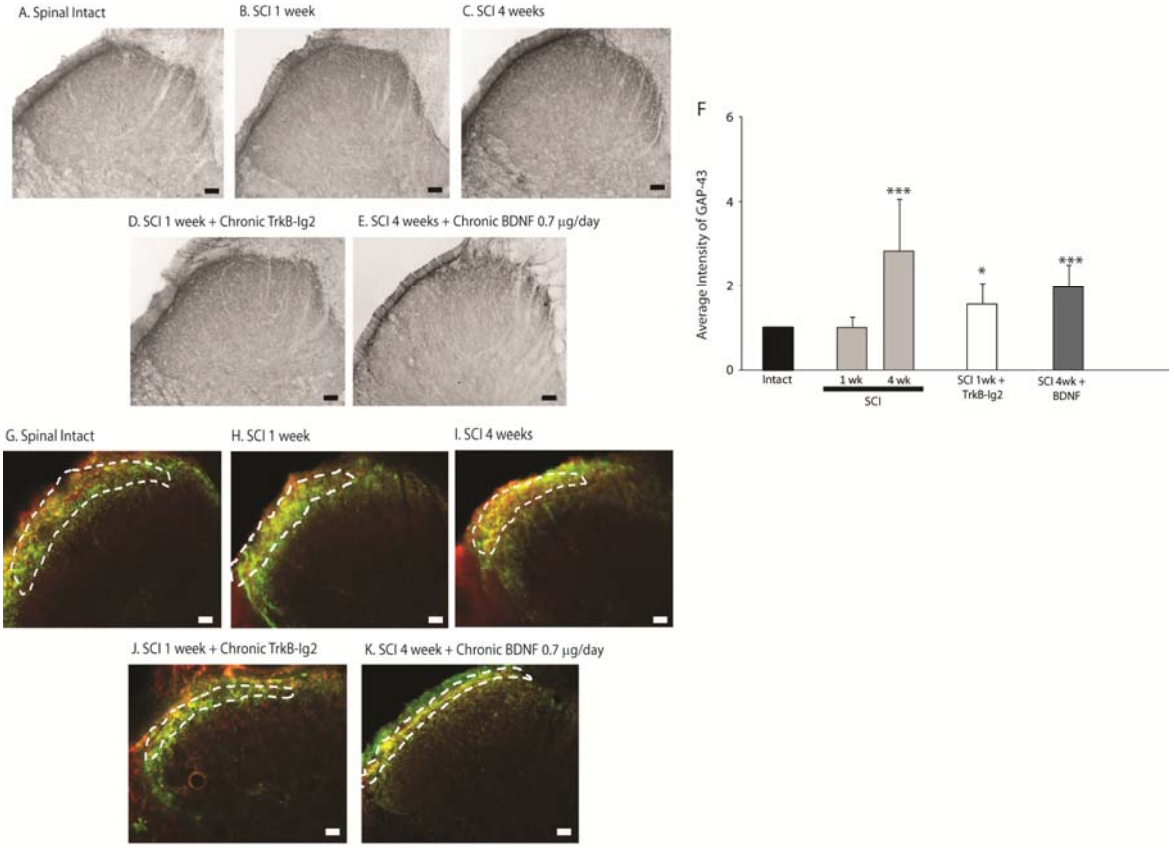

Figure 6. (A-F) Photomicrographs showing GAP-43 expression in the L5-L6 spinal cord segment of spinal intact (A), 1 week (B) and 4 weeks (C) SCT animals, 1 week SCT animals treated with chronic TrkB-Ig$_2$ and 4 weeks SCT animals treated with chronic saline (D) or BDNF (E; 0.7 µg/day). Scale bar=100 µm. D ! t-no ǿ I·ã LJNŜŘ2Y ƛyI·yŭtê ŜÈLJNŜãäŜŘ ƛy ŭKŜ ãdzLJŜNŧIÕƛI·f fI·Y ƛyI·Ŝ 2Ŧ ŭKŜ Ř2NãI·f K2NyΣ ǿƛŭK I· ŭƛY ŜŘŜLJŜyŘŜyŭ ƛyÕNŜI·ãŜ ó ! -/ŭΣ ƛyŘƛÕI·ŭƛyƎ ŭKŜ 2ÕÕdzNNŜyÕŜ 2Ŧ ŦƛŌNŜ ãLJN2dzŭƛyƎΦ /KN2yƛÕ ãI·fIyŜ ŘƛŘ yƶú I·ŦŦŜŭÕ D ! tno ŜÈLJNŜãäƛ2y ó5ŭΣ ǿKƛÕK ǿI·ã ãƛƎyŧƛÕI·yŭtê ƛyÕNŜI·ãŜŘ Ŧ2ff2ǿƛyƎ I·ŘY ƛyŧãŭNI·ŭƛ2y 2Ŧ ¢Nŧŧ . -LƎH Ŧ2N м ǿŜŜŧã óCΦ **(G) Histogram depicting the mean intensity of GAP-43 expression in the L5-L6 segment.** ¢KŜ ãŭN2yƎŜãŭ ƛyŭŜyãƛŭê 2Ŧ D ! t-no ǿI·ã 2ōãŜNØŜŘ ƛy Ŧ2dzN ǿŜŜŧã {/¢ I·yƛY I·fãΣ {/¢ I·yƛY I·fã ŭNŜI·ŭŜŘ ǿƛŭK ¢Nŧŧ . -LƎH I·yŘ ƛy {/¢ I·yƛY I·fã ÕKN2yƛÕI·ffê ŭNŜI·ŭŜŘ ǿƛŭK . 5bC óFLJF л Φлр ØŜNãdzã ãLJƛyI·f ƛyŭI·ÕŭΦ **(H-L) GAP-43 expression colocalization with CGRP neuropeptide in the dorsal horn. Scale bar = 20 µm.** LY I·ff ŜÈLJŜNƛY Ŝyŭ I·f ƎN2dzLJãΣ ŭKŜNŜ ǿI·ã I· ãŭNƛÕŭ Õ2-f2ÕI·fIÎ I·ŭƛ2y ōŜŭǿŜŜy D ! t-no I·yŘ /Dwtφ

Discussion

I ŜNŨΣ ǿŜ ƛyØŜãŭƛƎI·ŭŜŘ ŭKŜ Õ2yŭNƛōdzŭƛ2y 2Ŧ . 5bC ŭ2 ŭKŜ ŜY ŜNŦŜyÕŜ I·yŘ YI·ƛyŭŜyI·yÕŜ 2Ŧ b5hφ hdzN ŦƛyŘƛyƎã ŘŜY2yãŭNI·ŭŜ ŭKI·ŭ . 5bC LJfI·èã I· ŧŜè N2fŜ ƛy ŜãŭI·ōfIãKŜŘ b5hΣ I·ã Ŧ2dzyŘ ƛy ÕKN2yƛÕ {/L I·yƛY I·fãφ hy ŭKŜ 2ŭKŜN KI·yŘΣ 2dzN ŘI·ŭI· I·fã2 ƛyŘƛÕI·ŭŜã ŭKI·ŭ . 5bCΣ ŘdzNƛyƎ ãLJƛyI·f ãK2ÕŧΣ KI·ã I· LJN2ŭŜÕŭƛǿŜ N2fŜΣ LJNŜǿŜyŭƛyƎ 2N ŘŜfI·èƛyƎ ŭKŜ ŜY ŜNŦŜyÕŜ 2Ŧ b5hφ ¢KŜNŜŦ2NŜΣ 2dzN ãŭdzŘè ƛyŘƛÕI·ŭŜã ŭKI·ŭ . 5bC YI·è KI·ǿŜ ŘdzI·f NN2fŜ ƛy ōfI·ŘŘŜN ŦdzyÕŭƛ2y Ŧ2ff2ǿƛyƎ {/Lφ ! ŭ fŜI·ãŭ LJI·Nŭŭ 2Ŧ . 5bC ŜŦŦŜÕŭ YI·è 2ÕÕdzN ǿƛI· Y2ŘdzfI·ŭƛ2y 2Ŧ I·È2yI·f ãLJN2dzŭƛyƎ I·yŘ yŜdzNƛŭŜ ƎN2ǿŭK 2Ŧ ōfI·ŘŘŜN ãŜyã2Nè I·ŦŦŜNŜyŭãφ

BDNF is involved in chronic SCI-induced bladder dysfunction

² Ŝ 2ōãŜNØŜŘ ŭKI·ŭ ŭKŜ ŘŜØŜf2LJY Ŝyŭ 2Ŧ ōfI·ŘŘŜN ŘèãŧdzyÕŭƛ2y ǿI·ã I·ÕÕ2YLJI·yƛŜŘ õè ŭKŜ dzLJNŜ3dzfI·ŭƛ2y 2Ŧ ãLJƛyI·f . 5bC ŜÈLJNŜãäƛ2yΣ ãdz ƎƎŜãŭƛyƎ ŭKI·ŭ . 5bC ƛã fIyŢŜŘ ŭ2 b5hφ ¢2 Õ2yŦƛNY ŭKƛãΣ . 5bC ãŜIjdzŜãŭNI·ŭƛ2y ǿI·ã LJŜNŦ2NY ŜŘ ŘdzNƛyƎ Õèãŭ2Y ŜŭNè ƛy п ǿŜŜŢã {/L NI·ŭãφ ² Ŝ Ŧ2dzyŘ I· Ř2ãŝ-ŘŜLJŜyŘŜyŭ ƛYLJN2ǿŜY Ŝyŭ 2Ŧ ōfI·ŘŘŜN ŦdzyÕŭƛ2yΣ ǿƛŭK I· ãdzōãŭI·yŭƛI·f NŜŘdzÕŭƛ2y ƛy ŭKŜ ŦNŜIjdzŜyÕè I·yŘ I·YLJfƛŭdzŘŜ 2Ŧ ōfI·ŘŘŜN NŜŦfŜÈ Õ2yŭNI·Õŭƛ2yãφ [ƛŢŜǿƛãŜΣ I·ÕdzŭŜ . 5bC ãŜIjdzŜãŭNI·ŭƛ2y I·fã2 LJN2ŘdzÕŜŘ I· ãǿƛŦŭ ƛYLJN2ǿŜY Ŝyŭ 2Ŧ ōfI·ŘŘŜN ŦdzyÕŭƛ2y ƛy NI·ŭã ǿƛŭK Õèãŭƛŭãó CNƛI·ã Ŝŭ I·fΦΣ нлмоύΦ ¢KŜ NŜI·ã2y Ŧ2N 2dzN 2ōãŜNØI·ŭƛ2yã YI·è NŜfI·ŭŜ ŭ2 ƛyI·ÕŭƛØI·ŭƛ2y 2Ŧ ãŜÕ2yŘ-2NŘŜN ySdzN2yãφ . 5bC ãã I· LJ2ŭŜyŭ I·ÕŭƛØI·ŭ2N 2Ŧ ŭKŜ 9wY LJI·ŭKǿI·è ƛy ãLJƛyI·f ySdzN2yã ótŜÎŜŭ Ŝŭ I·fΦΣ нллнοТ [ŜØŜN Ŝŭ I·fΦΣ нллоοТ {I·fã2 Ŝŭ I·fΦΣ нллтТ a ŜNƛƎKƛ Ŝŭ I·fΦΣ нллуύ ǿKƛÕK I·y ŜI·NŧêãŭdzŘè ŘŜY2yãŭNI·ŭŜŘ KI·ã I· ŢŜè N2fŜ ƛy ŭKŜ ŜY ŜNŦŜyŘŜ 2Ŧ b5h ó/NdzÎ Ŝŭ I·fΦΣ нллсύΦ

Role of BDNF during the period of spinal shock

. 5bC ǿI·ã I·fNŜI·Rè ƛyÕNŜI·ãŜŘ м ǿŜŜŢ I·ŦŭŜN ãLJƛyI·f Õ2NŘ ŭNI·yãŜÕŭƛ2yφ 5dzNƛyƎ ŭKƛã ŦƛNãŭ ǿŜŜŢΣ Õèãŭ2Y ŜŭNè ãK2ǿŜŘ I· ŭ2ŭI·f I·ōãŜyÕŜ 2Ŧ ŘŜŭNdzã2N Õ2yŭNI·Õŭƛ2yãφ ¢I·ŢƛyƎ ŭKƛã ŦƛyŘƛyƎ I·yŘ ŭKŜ LJNŜØƛ2dzã 2ōãŜNØI·ŭƛ2yã ƛyŭ2 Õ2yãƛŘŜNI·ŭƛ2yΣ ǿŜ KèLJ2ŭKŜãƛÎŜŘ ŭKI·ŭ . 5bC ãŜIjdzŜãŭNI·ŭƛ2y Õ2dzfŘ LJNŜØŜyŭ ŭKŜ I·LJLJŜI·NI·yÕŜ 2Ŧ b5hφ Lŭ ãK2dzfŘ ōŜ NŜÕI·ffŜŘ ŭKI·ŭ I· ãƛY ƛfI·N KèLJ2ŭKŜãƛã KI·ã ōŜŜy NI·ƛãŜŘ õè {ŜŢƛ Ŝŭ I·f ǿK2 ãK2ǿŜŘ ŭKI·ŭ ãŜIjdzŜãŭNI·ŭƛ2y 2Ŧ bDCΣ ƛyŭƛI·ŭŜŘ ŭŜy ŘI·èã I·ŦŭŜN ãLJƛyI·f Õ2NŘ ƛyzdzNèΣ NŜŘdzÕŜŘ b5h I·yŘ 5{5 ó{ŜŢƛ Ŝŭ I·fΦΣ нллнТ

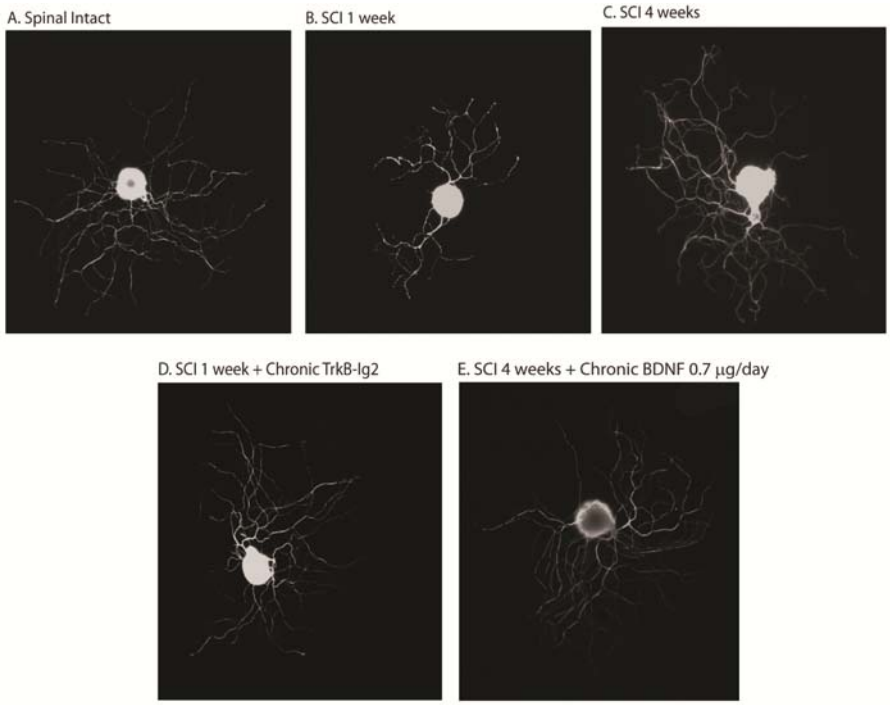

Figure 7. (A-E) Photomicrographs showing beta3-tubulin expression of L5-S1 DRGs neurons in culture. Scale bar=20 µm. Neurons from one week SCI animals (B) presented shorter neurites when compared to cell from spinal intact animals (A). In cells obtained from four weeks SCI animals (C) neurites were long and ramified. (D) Neurons from one week SCI animals treated with chronic TrkB-Ig_2 had long neurites, which were extremely ramified. Similar observations were found in DRG neurons from 4 week SCI rats receiving chronic BDNF (E).

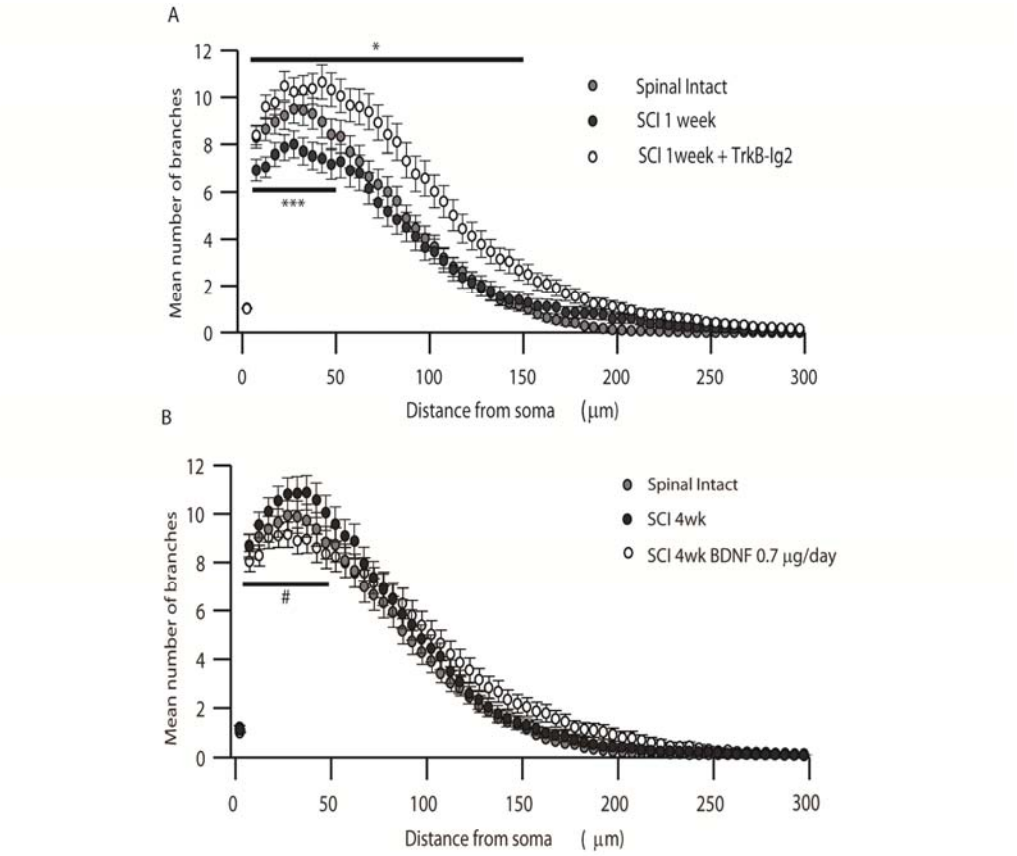

Figure 8. (A-B) Graphs indicating the correlation between the mean number of branches with the distance from the soma (µm). (A) One week SCI animals treated with TrkB-Ig_2 presented an higher number of branches in short and long distances from the soma (until 200 µm) when compared to spinal intact and one week SCI animals (*p<0.05). (B) Four weeks SCI animals registered an increase in the number of branches until 60 µm from the soma, when compared to spinal intact and four weeks SCI animals treated with BDNF. In contrast, four week SCI animals treated with chronic BDNF presented a significant higher number of branches from 110 µm to 200 µm (*p<0.05 versus spinal intact and four weeks SCI animals).

Table 2. aŜI·y ΰ2ΰÍ·f ySdzNƛΰŜ tŜySΨΰK ógY ú I·yŘ YŜI·y ă2Y I· I·NŜI· ógY[H]ú 2Ŧ ÕdzfΰdzNŜŘ 5wDă ÕŜffã 2Ŧ ălJƛyÍ·f ƛyΰÍ·Õΰ I·yΛY I·fãΣ 2yŜ I·yŘ ØŜŜ]ă {/¢ I·yΛY I·fãΣ 2yŜ ØŜŜ] {/L I·yΛY I·fã ΰNŜI·ΰŜŘ ØƛΰK ÕKN2yΛÕ ¢N] .-LƎH I·yŘ Ŧ2dzN ØŜŜ]ă {/L I·yΛY I·fã ΰNŜI·ΰŜŘ ØƛΰK ÕKN2yΛÕ .5bC⊕ /2yÕŜNyΛyƎ ΰKŜ ySdzNƛΰŜ tŜySΨΰKΣ ÕŜffã FN2Y 2yŜ ØŜŜ] {/L I·yΛY I·fã ΰNŜI·ΰŜŘ ØƛΰK ¢N] .-LƎH LJNŜăŜyΰŜŘ I· KƛƎKŜN ÕI·LJI·ÕΛΰè 2Ŧ ƎN2ØΰK óFLJFл⊕lp ØŜNădză 2y ØŜŜ] {/Lΰ⊕ /Ŝffã FN2Y Ŧ2dzN ØŜŜ]ă {/L I·yΛY I·fã LJNŜăŜyΰŜŘ I·y ƛyÕNŜI·ăŜ 2Ŧ ΰKŜ ă2Y I· I·NŜI· óFLJFл⊕lp ØŜNădză ălJƛyÍ·f ƛyΰÍ·ÕΰŤ [LJFл⊕lp ØŜNădză Ŧ2dzN ØŜŜ] {/L I·yΛY I·fã ΰNŜI·ΰŜŘ ØƛΰK ÕKN2yΛÕ .5bCŦ [9]LJFл⊕lp ØŜNădză м ØŜŜ] {/L I·yΛY I·fã ΰNŜI·ΰŜŘ ØƛΰK ÕKN2yΛÕ ¢N] .-LƎHΰ⊕

	Spinal intact	*SCI 1 week*	*SCI 1 week + chronic TrkB-Ig2*	*SCI 4 weeks*	*SCI 4 weeks + chronic BDNF*
Mean neurite length (μm)	мтно⊕оҕмпл⊕о	мрпр⊕тҕмро⊕оҒ	нолт⊕оҕмфн⊕т	муфн⊕фҕмоу⊕н	нлнл⊕пҕмро⊕о
Mean soma área (μm²)	моуо⊕лҕмрн⊕н	ммуу⊕нҕст⊕ф	фтс⊕уҕтл⊕мҒ	мнот⊕пҕст⊕ф[IIЭ]	ммлн⊕тҕоф⊕нҒ

{Ŝ]ƛ Ŝΰ I·f⊕Σ нллпΰ⊕ {dzNLJNƛăƛyƎfèΣ .5bC ăŜIjdzŜăΰNI·ΰƛ2y ŘdzNƛyƎ ΰKŜ ăLJƛyÍ·f ăK2Õ] LJŜNƛ2Ř ƛyŘdzÕŜŘ ΰKŜ ŜI·NfèŜ SY ŜNƎŜyÕŜ 2Ŧ b5h⊕ {ƛY ƛfI·N NŜădztΰă KI·ØŜ ōŜŜy Ŧ2dzyŘ ŜtăŜ⊕KŜNŜ⊕ ¦ăƛyƎ I· Y 2ŘŜf 2Ŧ ŘzNăI·f N22ΰ ƛy⊕dzNè Σ wI·YŜN I·yŘ Õ2ø2N]ŜNă ØŜNƛŦΛŜŘ ΰKI·ΰ .5bC ăŜIjdzŜăΰNI·ΰƛ2y NŜădztΰŜŘ ƛy 2LJLJ2ăƛΰŜ ŜŦŦŜÕΰă ŘŜLJŜyŘΛyƎ 2y ØKŜΰKŜN ƛΰ ø I·ă LJŜNŦ2NYŜŘ ÕKN2yΛÕI·ffè I·yŘ ƛyΰΛI·ΰŜŘ ƛYYŜŘΛI·ΰŜfè I·ŦΰŜN fŜăƛ2y 2N 2yfè ōè I· ăƛyƎfŜ ƛyΰNI·ΰKŜÕI·f ō2fdză I·ΰ fI·ΰŜN ăΰI·ƎŜă ówI·YŜN Ŝΰ I·f⊕Σ нллтú⊕ I ŜNŜΣ 2yŜ ÕI·y ƛyŦŜNΣ I·ƎI·ƛyăΰ 2dzN ƛyΰΛI·f KèLJ2ΰKŜăΛăΣ ΰKI·ΰ ΰKŜ ƛyÕNŜI·ăŜ 2Ŧ ălJƛyÍ·f .5bC ŘdzNΛyƎ ŜI·NfèŜ ăΰI·ƎŜă 2Ŧ {/L YI·è KI·ØŜ I· LJN2ΰŜÕΰΛØŜ N2fŜ 2y ōfI·ŘŘŜN Ŧdzyΰ2y⊕

¢2 ŦdzNΰKŜN Õ2yŦƛNY ΰKƛăΣ ØŜ LJN2Y2ΰŜŘ .5bC I·ŘY ƛyƛăΰNI·ΰƛ2y ΰ2 {/L I·yΛY I·fã⊕ ¢NŜI·ΰYŜyΰ ØI·ă Õ2yΰƛyΰz2dză I·yŘ ƛyΰΛI·ΰŜŘ ƛYYŜŘΛI·ΰŜfè I·ŦΰŜN Õ2NŘ ƛy⊕dzN⊕è ¢KŜ Y2ăΰ NŜfŜØI·yΰ ŦƛyŘΛyƎ ØI·ă ΰKI·ΰ I·ŘY ƛyƛăΰNI·ΰƛ2y 2Ŧ лфт µƎkŘI·è 2Ŧ .5bC Ŧ2N п ØŜŜ]ă LJN2ŘdzÕŜŘ I· ălƎyƛŦΛÕI·yΰ ŘŜÕNŜI·ăŜ 2Ŧ ŦNŜIjdzŜyÕè Σ I·YLJfΰΰdzŘŜΣ LJŜI·] LJNŜăădzNŜ I·yŘ ! ¦ /Σ Õ2yŦΛNYƛyƎ ΰKŜ LJN2ΰŜÕΰΛØŜ N2fŜ ΰKI·ΰ .5bC YI·è KI·ØŜ ΰ2 I·ΰΰŜyΰzI·ΰŜ ΰKŜ ŜYŜNƎŜyÕŜ I·yŘ YI·ƛyΰŜyI·yÕŜ 2Ŧ b5h⊕ hyŜ KèLJ2ΰKŜăΛă ƛă ΰKI·ΰ ΰKƛă ÕI·y 2ÖÕdzNNŜŘ Ø⅄I· LJ2ΰŜyΰƛI·ΰƛ2y 2Ŧ ΰKŜ D! .!ŜNƎƛÕ yŜdzN2ΰNI·yãYƛăăƛ2y⊕ .ŜÕI·dzăŜ D! .!ŜNƎƛÕ yŜdzN2yã ŜÈLJNŜăă ¢N] . NŜÕŜLJΰ2NΣ ΰKŜ ƛyÕNŜI·ăŜŘ I·Y2dzyΰ 2Ŧ .5bC I·ΰ ΰKŜ ălJƛyÍ·f Õ2NŘ YI·è KI·ØŜ ădzŦŦƛÕŜŘ ΰ2 ƛyÕNŜI·ăŜ D! .! NŜfŜI·ăŜ ótŜÎŜΰ Ŝΰ I·f⊕Σ нллнI·T [ŜØŜN Ŝΰ I·f⊕Σ нллоI·T .I·NŘ2yƛ Ŝΰ I·f⊕Σ нллтú⊕ hyŜ ăK2dzfŘ NŜÕI·ff ΰKI·ΰ ΰKƛă yŜdzN2ΰNI·yãYƛăăƛ2y KI·ă I·tNŜI·Ře õŜŜy Ŧ2NØI·NŘŜŘ I·ă I· LJ2ΰŜyΰƛI·f ΰKŜNI·LJŜdzΰƛÕ YŜI·ădzNŜ Ŧ2N b5h óaƛèI·Î I·ΰ2 Ŝΰ I·f⊕Σ нллуT aƛèI·Î I·ΰ2 Ŝΰ I·f⊕Σ нллфú⊕ I2ØŜØŜNΣ ΰKŜ Ř2ălƎŜ 2Ŧ .5bC õŜƛyƎ I·ŘY ƛyăΰŜNŜŘ ƛă ÕNΛΰƛÕI·f I·ă ΰKŜ KƛƎKŜăΰ Ř2ăŜ ΰŜăΰŜŘ ŘΛŘ yƎΰ I·ŦŦŜÕΰ õfI·ŘŘŜN Řèă⊕dzyÕΰƛ2y⊕ Lΰΰ ƛă ΰŜY LJΰƛyƎ ΰ2 ălJŜÕdzfI·ΰŜ I·õ2dzΰ I· LJ2ăăƛõfŜ ΰKŜNI·LJŜdzΰƛÕ I·LJLJŦƛÕI·ΰƛ2y 2Ŧ .5bC ƛy {/L LJI·ΰŜyΰă⊕ I2ØŜØŜNΣ ΰŜ ăK2dzfŘ õŜ yƎΰŜŘ ΰKI·ΰ I· KƛƎKŜN Ř2ăŜ 2Ŧ ΰKƛă b¢ ŘΛŘ yƎΰ LJN2ŘdzÕŜ I·yè ƛY LJN2ØŜYŜyΰ 2Ŧ õfI·ŘŘŜN ⊕dzyÕΰƛ2y⊕ !ΰ LJNŜăŜyΰ ØŜ ÕI·yƎƎΰ Ŧ2NØI·NŘ I· Õ2yÕfdzăƛØŜ ŜÈLJfI·yI·ΰƛ2y Ŧ2N ΰKŜăŜ ŦƛyŘΛyƎă õdzΰ ΰŜ YI·è õŜ LJ2ăăƛõfŜ ΰKI·ΰ ΰKŜ ŜÈÕŜăă 2Ŧ .5bC I·ŘYƛyƛăΰNŜŘ Õ2dzfŘ I·ÕΰƛØI·ΰŜ yƎy-ălJŜÕƛŦƛÕ b¢ NŜÕŜLJΰ2Nă I·yŘκ2N 2ΰKŜN ƛyΰNI·ÕŜffdzfI·N ăƛƎyI·ffƛyƎ LJI·ΰKØI·èă ƛy ălJƛyI·f ySdzN2yã⊕

Mechanisms modulating BDNF action on bladder function

tfI·ăΰÕ NŜ2NƎI·yΛÎI·ΰƛ2y 2Ŧ ΰKŜ ySdzN2yI·f ÕΛNÕdzΰΰă Ǝ2ØŜNyΛyƎ ōfI·ŘŘŜN Ŧdzyΰΰƛ2y KI·ă ōŜŜy Ŧ2NØI·NŘŜŘ I·ă ΰKŜ ÕI·dzăŜ 2Ŧ ōfI·ŘŘŜN Řèă⊕dzyÕΰƛ2y Ŧ2ff2ØƛyƎ {/L óŘŜ DN2I·ΰ Ŝΰ I·f⊕Σ мффлT ±ΛÎÎI·NŘΣ нллсú 2N ōfI·ŘŘŜN 2dzΰtSΰ 2ōăΰNdzÕΰƛ2y ó.hhú ó{ΰŜŜNă Ŝΰ I·f⊕Σ мффмT DI·ōŜffI· Ŝΰ I·f⊕Σ мффнT ¢dzΰΰtŜ I·yŘ {ΰŜŜNăΣ мффнú⊕ 9I·Nfè ăΰdzŘΛŜă KI·ØŜ ŘŜY2yãΰNI·ΰŜŘ ΰKI·ΰ ō2ΰK Õ2yŘΛΰƛ2yã KI·ØŜ I·y dzLJNŜƎdzfI·ΰƛ2y 2Ŧ bDC I·ăă2ÕΛI·ΰŜŘ ΰKI·ΰ fŜI·Ră ΰ2 KèLJŜNΰΰN2LJKè 2Ŧ YI·?2N LJŜtØΛÕ ƎI·yƎfΛ2y I·yŘ Ř2NăI·f N22ΰ ƎI·yƎfΛ2y ySdzN2yã ó{ΰŜŜNă Ŝΰ I·f⊕Σ мффмT {ΰŜŜNă Ŝΰ I·f⊕Σ мффсú I·yŘ ălJN2dzΰƛyƎ 2Ŧ ÕŜyΰNI·f ōNI·yÕKŜă 2Ŧ ōfI·ŘŘŜN ăŜyã2Nè I·ŦŦŜNŜyΰă ó±ΛÎÎI·NŘΣ мфффú⊕ I ŜNŜΣ ØŜ I·fă2 2ōăŜNØŜŘ ălJN2dzΰƛyƎ 2Ŧ ăŜyã2Nè õ2ΰK ŘdzNΛyƎ ΰKŜ yI·ΰdzNI·f LJN2ƎNŜăăΛ2y 2Ŧ ΰKŜ ŘΛăŜI·ăŜ I·yŘ I·ŦΰŜN .5bC ăŜIjdzŜăΰNI·ΰƛ2y ŘdzNΛyƎ ălJΛyI·f ăK2Õ] ! È2yI·f ƎN2ØΰK ØI·ă ŜØI·fdzI·ΰŜŘ ōè I·yI·fèăΛyƎ ΰKŜ ŜÈLJNŜăă2y 2Ŧ D! t-по Σ I· YI·N]ŜN 2Ŧ I·È2yI·f ƎN2ØΰK ó.Ŝy2ØΛΰÎ I·yŘ w2dzΰΰŜyÕŜNƎΣ мффтúΣ ØKΛÕK ƛyÕNŜI·ăŜŘ 2ØŜN ΰΛYŜ ƛy ΰKŜ ădzLJŜNŦΛÕΛI·f fI·YƛyI·Ŝ 2Ŧ ΰKŜ Õ2NŘΣ I·yŘ Õ2yŦΛNYƛŜŘ ƛy ÕŜff ÕdzfΰdzNŜ I·ăăI·èăΣ ΰKI·ΰ ŘŜY2yãΰNI·ΰŜŘ ΰKI·ΰ yŜdzNƛΰŜ 2dzΰƎN2ØΰK I·yŘ ōNI·yÕKΛyƎ Ŧ2ff2ØŜŘ ΰKŜ LJI·ΰΰŜNY 2Ŧ ălJƛyI·f ŜÈLJNŜăăΛ2y 2Ŧ D! t-по⊕ ¢KŜ NŜI·ă2y dzyŘŜNfèΛyƎ ălJN2dzΰƛyƎ 2Ŧ ØΛăÕŜNI·f I·ŦŦŜNŜyΰă ŘdzNΛyƎ ΰKŜ LJN2ƎNŜăΛ2y 2Ŧ ΰKŜ ŘΛăŜI·ăŜ YI·è õŜ NŜfI·ΰŜŘ ØΛΰK ΰKŜ NŜLJ2NΰΰŜŘ ŜfŜØI·ΰƛ2y 2Ŧ ălJƛyI·f bDC fŜØŜfă ó{Ŝ]ƛ Ŝΰ I·f⊕Σ нллнT .N2øy Ŝΰ I·f⊕Σ нллпΰΣ ΰKI·ΰ ăΰΛYdzfI·ΰŜă ΰKŜ *in vitro* ƎN2ØΰK 2Ŧ 5wD ySdzN2yã óDI·ØI·ÎÎƛ Ŝΰ I·f⊕Σ мфффú⊕ LyÕNŜI·ăŜŘ ŘŜyãΛΰè 2Ŧ ăŜyã2Nè I·ŦŦŜNŜyΰă YI·è fŜI·Ř ΰ2 ƛyÕNŜI·ăŜŘ NŜfŜI·ăŜ 2Ŧ .5bCΣ fŜI·ŘƛyƎ ΰ2 Ř2øyãΰNŜI·Y I·ÕΰΛØI·ΰƛ2y 2Ŧ 9wYΣ øKΛÕK KI·ă ōŜŜy LJNŜØΛ2dzătfè fΛy]ŜŘ ΰ2 õfI·ŘŘŜN Řèă⊕dzyÕΰƛ2yƛy {/L NI·ΰă ó/NdzÎ Ŝΰ I·f⊕Σ нллсú⊕ !ă ŜÈLJŜÕΰŜŘ óhyŘI·NÎI· Ŝΰ I·f⊕Σ нллоΰΣ øŜ 2ōăŜNØŜŘ Ŧdzff Õ2f2ÕI·fΛÎI·ΰƛ2y 2Ŧ ΰKΛă ƎN2ØΰK YI·N]ŜN øΛΰK /Dwt Σ ƛyŘΛÕI·ΰƛyƎ ΰKI·ΰ I·È2yI·f ălJN2dzΰƛyƎ 2Ŧ õfI·ŘŘŜN LJŜLJΰΛŘŜNƎΛÕ ăŜyã2Nè I·ŦŦŜNŜyΰă ƛă ΰKŜ]Sè ŜØŜyΰ Ŧ2N õfI·ŘŘŜN Řèă⊕dzyÕΰƛ2y·I·ŦΰŜN {/L ó½ƛyÕ] Ŝΰ I·f⊕Σ нллтú⊕

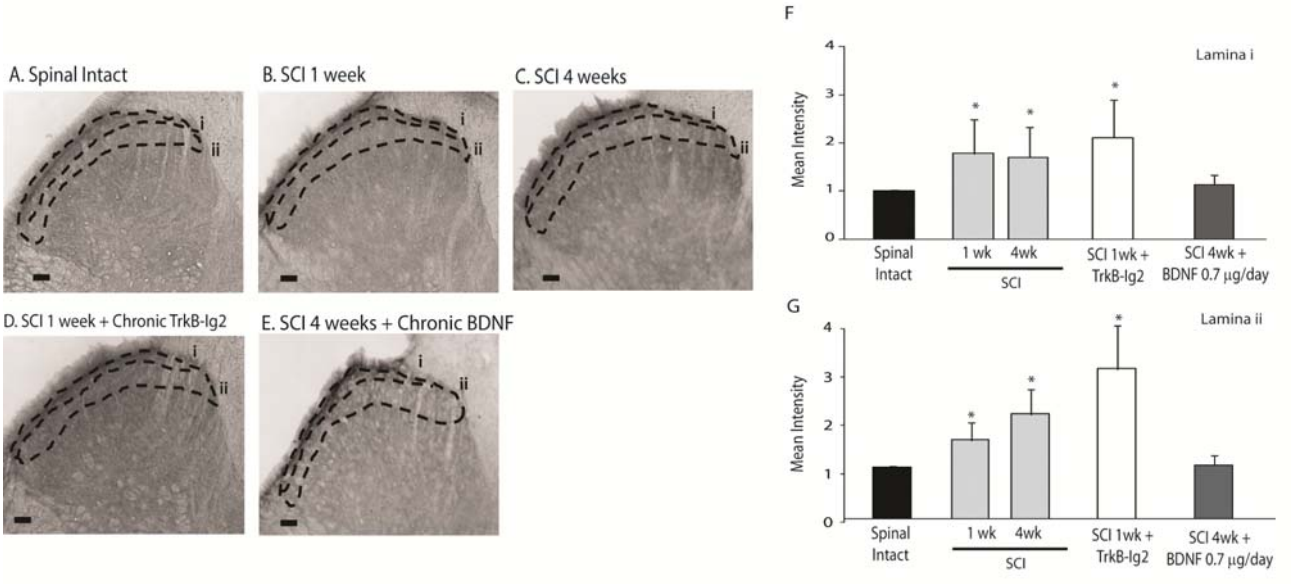

Figure 9. (A-E) Representative photomicrographs depicting JNK expression in L5-L6 spinal cord segment of spinal intact, one week and four weeks SCI animals, one week SCI animals treated with chronic TrkB-Ig$_2$ and four weeks SCI animals treated with chronic BDNF. Scale bar = 100 µm. tK2ăLJK2₩bY ƛYˇYdzyŽ₦ŜI٠Ŏΰƛ2yˇ ø I٠ă ŧŽdzyŘ ƛyˇŘ2₦ăI٠f K2₦yΣ LJI٠₦ΰƛŎdzfI٠₦fê ƛyˇƛyˇfI٠YˇƛyI٠Ŝ L I٠yŘ LL¢ **(F, G) Graph of bars showing the mean intensity of JNK expression in laminae I (F) and laminae II (G).** ! yI٠fêăƛă 2ŧ ΰKŜ ƛyΰŜyãƛΰê 2ŧ LJK2ăLJK2₩bY ƛYˇYdzyŽ₦ŜI٠Ŏΰƛ2yˇăK2øŜŘ I٠ ΰƛYˇŜ-ŘŜLJŜyŘŜyΰ ƛyŎ₦ŜI٠ăŜ ƛyˇ ΰKŜ ăLJƛyI٠f ŎŽ₦ŘŽŧ { /L ₦I٠ΰă I٠yŘ ƛyˇŎŽ₦ŘŽŧ ăŜŎΰƛ2yã ŦN2Y м øŜŜ] { /L I٠yIYˇI٠fã ΰ₦ŜI٠ΰŜŘ øƛΰK ŎK₦2yƛŎ ¢₦] . -LƎ„¢ ¢Kƛă øI٠ă Y2₦Ŝ ŜØƛŘŜyΰ ƛyˇfI٠YˇƛyI٠₦ LL óDΰ ΰKI٠yˇ ƛyˇfI٠YˇƛyI٠ L óCΰ óFLJ꜀лnp ØŜ₦ădzã ăLJƛyI٠f ƛyΰI٠Ŏΰΰ¢ tK2ăLJK2₩bY ƛYˇYdzyŽ₦ŜI٠Ŏΰƛ2yˇƛyˇŎŽ₦ŘŽŧ {/L ₦I٠ΰă ΰ₦ŜI٠ΰŜŘ . 5bC ăŜIjdzŜăΰ₦I٠ΰƛ2yˇ øI٠ă ăƛYˇƛfI٠₦ Ŭ2 ăLJƛyI٠f ƛyΰI٠Ŏΰ I٠yƛYˇI٠fãΣ I٠fΰK2dzƎK ƛΰ ΰŜyŘŜŘ Ŭ2 ƛyŎ₦ŜI٠ăŜ¢

[ƛ]Ŝøƛă ŜΣ ăLJ₦2dzΰƛyƎ 2ŧ LJŜLJΰƛŘŜ₦ƎƛŎ I٠ŦŦŜ₦ŜyΰăI٠fΰŜ₦ ŎŽYˇLJfŜΰŜ {/L ăă I٠fãƨ I٠ăăƨŎƛI٠ΰŜŘ øƛΰK I٠dzΰƨyŽYˇƛŎ ŘèăK₦ŜŦfŜÈƛI· óY₦ŜyÎ I٠yŘ ² ŜI٠ØŜ₦Σ мффуТ Y₦ŜyÎ Ŝΰ I٠f¢Σ мфффТ ² ŜI٠ØŜ₦ Ŝΰ I٠f¢Σ нллмú I٠ă I٠LJƨăΰΰƛØŜ ŎŽ₦₦ŜfI٠ΰƛ2yˇ ŏŜΰøŜŜyˇ KèLJŜ₦ΰŜyãƛ2yˇ I٠yŘ ăLJ₦2dzΰƛyƎ 2ŧ /Dwt-LJƨăΰΰƛØŜ I٠È2yã KI٠ă I٠f₦ŜI٠Řè ōŜŜyˇ ƛŘŜyΰƛŦfΛŜŘ ó/I٠YˇŜ₦2yˇŜΰ I٠f¢Σ нллсú¢

!yˇ ƛyΰ₦ƛƎdzƛyƎ 2ōăŜ₦ØI٠ΰƛ2yˇ øI٠ă ΰKI٠ΰ . 5bC ăŜIjdzŜăΰ₦I٠ΰƛ2yˇ ŜyKI٠yŎŜŘ I٠È2yI٠f Ǝ₦2øΰK¢ *In vitro* ăΰdzŘfƛŜă KI٠Ř I٠f₦ŜI٠Řè Ř2ŎdzYˇŜyΰŜŘ ΰKŜ ƛyKƛōƛΰ2₦è ŜŦŦŜŎΰă 2ŧ . 5bC 2yˇ bDC-Ř₦ƛØŜyˇ ySdz₦ƛΰŜ Ǝ₦2øΰK óDI٠ØI٠Î Îƛ Ŝΰ I٠f¢Σ мфффТ YƛYˇLJƛyã]ƛ Ŝΰ I٠f¢Σ мфффú¢ !fΰK2dzƎK y2ΰ YˇŜI٠ădz₦ŜŘ ƛyˇ ΰKŜ LJ₦ŜăŜyΰ ăΰdzŘèΣ ƛΰ ƛă ØŜ₦è fƛ]Ŝfè ΰKI٠ΰ ƛyˇ 2dz₦ {/L I٠yƛYˇI٠fã ΰKŜ I٠Yˇ2dzyΰ 2ŧ ăLJƛyI٠f bDC ø I٠ă ØŜ₦è KƛƎK I٠yŘ LJ₦2Yˇ2ΰŜŘ I٠È2yI٠f Ǝ₦2øΰK¢ øKƛŎK ø I٠ă Yˇ2₦ŜΣ ŜÈdzōŜ₦I٠yΰ I٠yŘ 2ŎŎdz₦₦ŜŘ ŜI٠₦fΛŜ₦ ƛyˇΰKŜ I٠ōăSyŎŜ 2ŧ ΰKŜ ƛyKƛōƛΰ2₦è LJ₦ŜăŜyŎŜ 2ŧ . 5bC¢

Molecular mechanisms governing afferent sprouting at dorsal horns

!yˇ ƛYˇLJ2₦ΰI٠yΰ ƛyΰ₦I٠ŎŜffdzfI٠₦ ăƛƎyI٠ffƛyƎ LJI٠ΰK ø I٠è ƛyØ2fØŜŘ ƛyˇ ySdz₦ƛΰŜ 2dzΰŜƎ₦2øΰK I٠yŘ Ŝf2yƎI٠ΰƛ2yˇ ƛă ΰKŜ Ŏ-Wdzyˇ b-ΰŜ₦YˇƛyI٠f]ƛyI٠ăŜ óWbYú LJI٠ΰK ø I٠è ¢Kƛă ăƛƎyI٠ffƛyƎ ŎI٠ăŎI٠ŘŜ ƛă 2yŜ 2ŧ ΰKŜ YˇŜYˇōŜ₦ă 2ŧ ΰKŜ Yˇƛΰ2ƎŜy-I٠ŎΰƛØI٠ΰŜŘ LJ₦2ΰŜƛyˇ]ƛyI٠ăŜă óa ! tYăú fI٠₦ƎŜ ŦI٠Yˇƛfê 2ŧ ăƛƎyI٠ffƛyƎ]ƛyI٠ăŜă ó ² ƛŘYˇI٠yyˇŜΰ I٠f¢Σ мфффТ W2Kyã2yˇ I٠yŘ [I٠LJI٠ŘI٠ΰΣ нллнТ Y₦ăăKyI٠ I٠yŘ bI٠₦JI٠yƎΣ нллуú¢ ¢KŜ I٠ŎΰƛØŜ Ŧ2₦Y 2ŧ WbY KI٠ă ΰ₦JI٠Řƛΰƛ2yI٠ffè ōŜŜyˇfƛyˇ]ŜŘ ΰ2 ySdz₦2yI٠f ŘŜƎŜySy₦JI٠ΰƛ2yˇ I٠ŦΰŜ₦ ăΰ₦ŜăăI٠yŘ ƛyˇdz₦è¢ I2øŜØŜ₦Σ ƛΰ øI٠ă ₦ŜŎŜyΰfê ŘŜY2yãΰ₦I٠ΰŜŘ ΰKI٠ΰ WbY I٠ŎΰƛØI٠ΰƛ2yˇ øI٠ă ₦ŜIjdz₦ŜŘ Ŧ2₦ I٠È2yˇŦ2₦YˇI٠ΰƛ2yˇ I٠yŘ Ǝ₦2øΰK óhfƛØI· Ŝΰ I٠f¢Σ нллсТ . I٠₦yI٠ΰ Ŝΰ I٠f¢Σ нлмлТ !ΰ]ƛyã2yˇ Ŝΰ I٠f¢Σ нлммúΣ ΰ2 ăΰI٠ōΛfƛÎŜ I٠yŘ Y2Řdzfl٠ΰŜ ΰKŜ YˇƛŎ₦2ΰdzŏdzfŜ Řèyl٠YˇƛŎă I٠ΰ ΰKŜ Ǝ₦2øΰK Ŏ2yŜ I٠yŘ ySdz₦ƛΰŜ ΰƛLJă ó. I٠₦yI٠ΰ Ŝΰ I٠f¢Σ нлмлú¢ ! ŎŎ2₦ŘƛyƎfêΣ øŜ Ŧ2dzyŘ ăΰ₦2yƎŜ₦ ŜÈLJ₦ŜăăƛYˇ 2ŧ LJK2ăLJK2WbY ƛyˇ ΰKŜ ŜÈLJŜ₦ƛYˇSyΰI٠f Ǝ₦2dzLJă ƛyˇ øKƛŎK I٠È2yI٠f ăLJ₦2dzΰƛyƎ øI٠ă YˇŽ₦Ŝ LJ₦2y2dzyŎŜŘ¢ ¢Kƛă ƛă ΰKŜ ăLJƛyI٠f f2ŎI٠ΰƛ2yˇ øKŜ₦Ŝ D!t-no ƛYˇYdzyŽ₦ŜI٠ŎΰƛØƛΰè øI٠ă I٠fãƨ ăΰ₦2yƎŜ₦Σ Ŏ2₦₦ŜăLJ2yŘƛyƎ ΰ2 ΰKŜ I٠₦ŜI٠ă 2ŧ ăLJ₦2dzΰƛyƎ 2ŧ ōfI٠ŘŘŜ₦ ăSyã2₦è I٠ŦŦŜ₦Ŝyΰăŧ LyΰŜ₦ŜăΰƛyƎfèΣ I٠ŘYˇƛyŋăΰ₦I٠ΰƛ2yˇ 2ŧ . 5bC I٠fãƨ LJ₦2Yˇ2ΰŜŘ ySdz₦2yI٠f ăLJ₦2dzΰƛyƎΣ I٠fōŜƛΰ ōŋI٠yŎKƛyƎ Ŧ2ffƨøŜŘ I٠ ŘƛŦŦŜ₦Ŝyΰ LJI٠ΰΰŜ₦yΣ øƛΰK Y2₦Ŝ ŋI٠YˇƛŦƛŜŘ ōŋI٠yŎKŜă ōŜƛyƎ 2ōăŜ₦ØŜŘ I٠ΰ fƨyƎŜ₦ ŘƛăΰI٠yŎŜă ŦN2Y ΰKŜ ŎŜff ō2Řè¢ Ly ΰKƛă ŎI٠ăŜΣ WbY I٠ŎΰƛØI٠ΰƛ2yˇ øI٠ă ăƛYˇƛfI٠₦ ΰƨ Ŏ2yΰ₦2fãΣ ƛyŘƛŎI٠ΰƛyƎ ΰKI٠ΰ . 5bC-YˇŜŘƛI٠ΰŜŘ I٠È2yI٠f ăLJ₦2dzΰƛyƎ øI٠ă WbY-ƛyŘŜLJŜyŘŜyΰ¢

Conclusions

Lyˇ Ŏ2yŎfdzăƛ2yΣ ΰKŜăŜ ŦƛyŘƛyƎă ƛŘŜyΰƛŦè . 5bC I٠ă I٠yˇ ƛYˇLJ2₦ΰI٠yΰ Y2Řdzfl٠ΰ2₦ 2ŧ b5h ŜYˇŜ₦ƎŜyŎŜ ōè ₦ŜƎdzfI٠ΰƛyƎ ăLJ₦2dzΰƛyƎ 2ŧ ăŜyã2₦è I٠ŦŦŜ₦ŜyΰăΣ ΰKŜ]Ŝè YˇŜŎKI٠yŋăY dzyŘŜ₦fèƛyƎ ΰKŜ ŜYˇŜ₦ƎŜyŎŜ 2ŧ ōfI٠ŘŘŜ₦ Řèăfdzyŏΰƛ2y¢ ² Ŝ LJ₦2LJ2ăŜ ΰKI٠ΰ ΰKŜ dzLJ₦ŜƎdzfI٠ΰƛ2yˇ 2ŧ ΰKƛă b¢ ŘdzΰƛyƎ ŘƛăŜI٠ăŜ LJ₦2Ǝ₦ŜăăƛΰyˇYI٠è ăŜ₦ØŜ I٠ă I٠yˇ I٠ΰΰŜYˇLJΰ ΰ2 Ŏ2yΰ₦2f ŜÈI٠ƎƎŜ₦I٠ΰŜŘ I٠È2yI٠f ăLJ₦2dzΰƛyƎ¢

I2ǿŜØŜNΣ 2yÕŜ ăLJN2dzΩʎyƎ KI·ă 2ŎÖdzNNNŜŘΣ I·ÖdzΩŜ .5bC ãŜIjdzŜăΩNI·ΩʎƵyˊYˊI·è ãŜNØŜ Ω2 ŘŜLJNŜăä õfI·ŘŘŜN KèLJŜNI·ÖΩʎØʎΩèΦ ¢KdzãΣ .5bC ʎă I·yˊ I·ΩΩNI·ÖΩʎyƎ ΩKŜNI·LJŜdzΩʎÖ Ω̈I·NƎŜΩ̈ Ω2 õŜ ŘʎŦŦŜNŜyΩʎI·ffè YˊI·yƵLJdzfI·ΩŜŘ I·Ω̈ ŘʎŦŦŜNŜyΩ̈ ăΩ̈I·ƎŜă 2Ŧ b5h ŜăΩ̈I·õfʎăKYˊŜyΩ̈Φ

Acknowledgements

LyΩ̈ʎI·f ŦʎyI·yÕʎI·f ădzLJLJ2NΩ̈ ÖI·YˊŜ ŦN2Yˊ Ly/2YˊõóCtt I9![¢I LJN2ŝŜÖΩ̈ y2 нноноπύΦ .ŁNJõI·NJI· CNʎI·ă ʎă ădzLJLJ2NΩ̈ŜŘ õè C/¢ - CdzyŘI·œń2 LJI·NJI· I· /ʎŞyÕʎI· Ŝ ¢ŜÖy2f2Ǝʎ I· ó{CwIκ.5ксоннркнллфύΦ ¢KŜ I·dzΩ̈K2NΩă ǿ2dzfŘ fʎ]Ŝ Ω̈2 I·Ö]y2ǿfŜŘƎŜ ΩKŜ ΩŜÖKyʎÖI·f ădzLJLJ2NΩ̈ 2Ŧ {ŞNƎʎ2 /I·NJØI·fK2-.I·NJN2ă㊠

List of references

!Ω̈]ʎyã2y tWΣ /K2 /IΣ II·yãŜyˊ awΣ DNŜŜyˊ {I óнлммύ !ÖΩ̈ʎØʎΩ̈è 2Ŧ I·ff WbY ʎă2Ŧ2NYˊă Ö2yΩ̈NΩ̈õdzΩ̈Să Ω̈2 ySdzNΩ̈Ω̈Ŝ ƎN2ǿΩ̈K ʎyˊ ăLJNJI·f ƎI·yƎfΩ̈yˊ ySdzN2yã㊠I ŜI·NΩʎyƎ NŜăŜI·NÖK нтуʏтт-ур㊠

.I·yŦʎŜfŘ aWΣ bI·ëf2N w[Σ w2ΩŜNΩ̈ă2yˊ !DΣ !ffŜyˊ{WΣ 5I·ǿõI·NyˊΣ 5Σ .NJI·Ře w[óнллмύ {LJŜÖʎŦʎÖʎΩ̈è ʎyˊ ¢N]Ł NŜÖŜLJΩ̈2NYySdzN2Ω̈N2LJKʎyˊ ʎyΩ̈ŜNI·ÖΩʎ2yãʏ Ω̈KŜ ÖNΩèăΩ̈I·f ăΩ̈NdzÖΩ̈dzNΩŜ 2Ŧ ¢N]Ł.-Řp ʎyˊ Ö2YˊLJfŜÈ ǿʎΩ̈K ySdzN2Ω̈N2LJKʎy-пкр㊠{Ω̈NdzÖΩ̈dzNΩŜ фʏммфм-ммфф㊠

.I·NŘ2yʎ WΣ DKʎNNʎ !Σ{I·fΩ2 /Σ tNJI·yŘʎyʎ aΣ aŜNΩ̈ƎKʎ ! óнллтύ .5bC-YˊŜŘʎI·Ω̈ŜŘ Yˊ2ŘdzfI·Ωʎyˊ 2Ŧ D!.! I·yŘ ƎfèÖʎyŜ NΩ̈ŜfŜI·ăŜ ʎyˊ Ř2NΩ̈ăI·f K2NΩ̈yˊ fI·YˊʎyI· LL ŦN2Yˊ LJ2ăΩ̈yI·Ω̈I·f NJI·Ω̈ă㊠ 5ŜØŜf2LJYˊŜyΩ̈I·f ySdzN2õʎ2f2Ǝè стʏфсл-фтр㊠

.I·NyI·Ω̈ aΣ 9yãfŜyˊ IΣ tN2LJăΩ̈ CΣ 5I·ØʎăWWΣ {2I·NŜă {Σ b2Ω̈KʎI·ă C óнлмлύ 5ʎăΩ̈ʎyÖΩ̈ N2f2Ŝă 2Ŧ Ö-WdzyˊbΩ̈ŜNYˊʎyI·f]ʎyI·ăŜ ʎă2Ŧ2NYˊă ʎyˊ ySdzNΩ̈ŜŜ ʎyΩ̈ʎI·Ω̈ʎ2yˊ I·yŘ Ŝf2yƎI·Ω̈ʎ2yˊ ŘdzNΩ̈ʎyƎ I·È2yI·f NΩ̈ƎŜyŜNI·Ω̈ʎ2y㊠ W bŜdzN2ăÖʎ олʏтулп-тумс㊠

.Ŝy2ǿʎΩ̈Î [LΣ w2dzΩ̈Ω̈ŜyΩ̈ŜNƎ ! óмффтύ D!t-поʏ I·yˊʎyΩ̈NΩʎyãʎÖ ŘŜΩ̈ŜNYˊʎyI·yΩ̈ 2Ŧ ySdzN2yI·f ŘŜØŜf2LJYˊŜyΩ̈I·f ŸdzNΩ̈ʎyƎ I·È2yI·f NΩ̈ƎŜyŜNI·Ω̈ʎ2y㊠ W bŜdzN2ăÖʎ олʏтулп-тумс㊠

.Ŝy2ǿʎΩ̈Î [LΣ w2dzΩ̈Ω̈ŜyΩ̈ŜNƎ ! óмффтύ D!t-поʏ I·yˊʎyΩ̈NΩʎyãʎÖ ŘŜΩ̈ŜNYˊʎyI·yΩ̈ 2Ŧ ySdzN2yI·f ŘŜØŜf2LJYˊŜyΩ̈I·f LJfI·ăΩ̈ʎÖʎΩ̈è ¢NΩ̈ŜyŘă ʎyˊ ySdzN2ăÖʎŜyÖŜă нлʏуп-фм㊠

.N2ǿyˊ !Σ wʎÖÖʎ a-WΣ ²ŜI·ØŜN [/ óнллпύ bDC YˊŜăăI·ƎŜ I·yŘ LJN2Ω̈ŜʎyˊŘʎăΩ̈NΩ̈ʎõdzΩ̈ʎ2yˊ ʎyˊ Ω̈KŜ ʎyΩ̈dzNΩ̈ŜŘ NJI·Ω̈ ăLJʎyI·f Ö2NΩ̈Ř㊠ 9ÈLJŜNΩ̈ʎYˊŜyΩ̈I·f bŜdzN2f2Ǝè мууʏммр-мнт㊠

/I·YˊŜN2yˊ !!Σ {YˊʎΩ̈K DaΣ wI·yŘI·ft 5/Σ .N2ǿyˊ 5wΣ wI·õÖKŜØăʎè !D óнллсύ DŜySΩ̈ʎÖ YˊI·yƵLJdzfI·Ω̈ʎ2yˊ 2Ŧ ʎyΩ̈NJI·ăLJʎyI·f LJfI·ăΩ̈ʎÖʎΩ̈è I·ŦΩ̈ŜNJ ăLJʎyI·f Ö2NΩ̈Ř ʎyΩ̈dzNΩ̈è I·fΩ̈ŜNΩ̈ă Ω̈KŜ ăŜØŜNΩ̈Ω̈Ŝ 2Ŧ I·dzΩ̈2y2YˊʎÖ ŘèăNΩ̈ŜŦfŜÈʎI·㊠ W bŜdzN2ăÖʎ нсʏнфно-нфон㊠

/NdzÎ /Σ /NdzÎ C óнлммύ {LJʎyI·f /2NΩ̈Ř LyΩ̈dzNΩ̈è I·yŘ .fI·ŘŘŜN 5ëăŦdzyÕΩ̈ʎ2yʏ bŜǿ LŘŜI·ă I·õ2dzΩ̈ I·yˊ hfŘ tN2õΩ̈ŜYˊ¢KŜ{ÖʎŜyΩ̈ʎŦʎÖ ² 2NΩ̈fŘWh¦wb![ммʏнмп-ноп㊠

/NdzÎ /5Σ aÖaI·K2yˊ {.Σ /NdzÎ C óнллсύ {LJʎyI·f 9wY I·ÖΩ̈ʎØI·Ω̈ʎ2yˊ Ö2yΩ̈NΩʎõdzΩ̈Să Ω̈2 Ω̈KŜ NŜƎfI·Ω̈ʎ2yˊ 2Ŧ õfI·ŘŘŜN Ŧdzyˊ Ö Ω̈ʎ2yˊ ʎyˊ ăLJʎyI·f Ö2NΩ̈Ř ʎyΩ̈dzNΩ̈ŜŘ NJI·Ω̈ă㊠ 9ÈLJ bŜdzN2f нллʏсс-то㊠

/NdzÎ /5Σ !ØŜfʎy2 !Σ aÖaI·K2yˊ {.Σ /NdzÎ C óнллрύ LyÖNΩ̈ŜI·ăŜŘ ăLJʎyI·f Ö2NΩ̈Ř LJK2ăLJK2NΩ̈ŜfI·Ω̈ʎ2yˊ 2Ŧ ŜÈΩ̈NJI·ÖŜffdzfI·N ăʎƎyI·f-NΩ̈Ǝdzfl·Ω̈ŜŘ]ʎyI·ăŜă YˊŜŘʎI·Ω̈Ŝă YˊʎÖΩ̈dzNΩ̈Ω̈ʎ2yˊ2ØŜNJI·ÖΩ̈ʎØʎΩ̈è ʎyˊNJI·Ω̈ă ǿʎΩ̈K ÖKN2yʎÖ õfI·ŘŘŜN ʎyŦfI·YˊYˊI·Ω̈ʎ2y㊠ ¢KŜ 9dzN2LJŜI·yˊ ƨ2dzNyI·f 2Ŧ yŜdzN2ăÖʎŜyÖŜ нмʏтто-тум㊠

de GroI·Ω̈ ²/Σ YI·ǿʎI·Ω̈I·yʎ aΣ IʎăI·YˊʎΩ̈ădz ¢Σ /KŜyƎ /[Σ aI· /tΣ ¢K2N YΣ {Ω̈ŜŜNΩ̈ă ²Σ w2LJLJ2f2 Ww óмффлύ aŜÖKI·yʎăYˊă dzyŘŜNΩ̈fèʎyƎ Ω̈KŜ NΩ̈ŜÖ2ǿŜNΩ̈è 2Ŧ dzNΩ̈ʎyI·NΩ̈è õfI·ŘŘŜN ŦdzyÕ-Ω̈ʎ2yˊ Ŧ2ff2ǿʎyƎ ăLJʎyI·f Ö2NΩ̈Ř ʎyƨdzNΩ̈è㊠ W !dzΩ̈2yˊ bŜNØ {ëăΩ̈ ол {dzLJLJfʏ{тм-тт㊠

ŘŜ DN2I·Ω̈ ²/{² óмффлύ !dzΩ̈2yƨYˊʎÖ NΩ̈ŜƎdzfI·Ω̈ʎ2yˊ 2Ŧ Ω̈KŜ dzNΩ̈ʎ-yI·NΩ̈è õfI·ŘŘŜN I·yŘ ăŜÈ 2NƎI·yã㊠ LyʏˊŜyΩ̈NI·f NΩ̈ŜƎdzfI·-Ω̈ʎ2yˊ 2Ŧ I·dzΩ̈2yƨYˊʎÖ ŦdzyÕΩ̈ʎ2yã㊠ ó[2ŜǿèŁ5{YΣ ŜŘΩ̈ΣLJLJ омл-ооо㊠ [2yŘ2yʏ hÈŦ2NŘ ¦yØŜNăʎΩ̈è tNΩ̈Ŝää㊠

CNΩ̈ŜyÕK W{Σ !yŘŜNΩ̈ă2y-9NΩ̈ăYˊI·yˊY5Σ {dzΩ̈Ω̈ŜN a óнлмлύ ²KI·Ω̈ Ř2 ăLJʎyI·f Ö2NΩ̈Ř ʎyƨdzNΩ̈è Ö2yãdzYˊŜNΩ̈ă ǿI·yΩ̈K ! NΩ̈ŜØʎŜǿ 2Ŧ ăLJʎyI·f Ö2NΩ̈Ř ʎyƨdzNΩ̈è Ö2yãdzYˊŜN LJNΩ̈2NΩ̈ʎŜă I·yŘ ySdzN2-LJN2ăΩ̈KŜăʎă ŦN2Yˊ Ω̈KŜ нллу ySdzNJI·f ʎyΩ̈ŜNŦI·ÖŜă Ö2y-ŦŜNΩ̈ŜyÕŜ㊠ bŜdzNΩ̈2Yˊ2ŘdzfI·Ω̈ʎ2yˊ ʏ ƨ2dzNyI·f 2Ŧ Ω̈KŜ LyΩ̈ŜN-yI·Ω̈ʎ2yI·f bŜdzNΩ̈2Yˊ2ŘdzfI·Ω̈ʎ2yˊ{2ÖʎŜΩ̈è моʏннф-ном㊠

CNΩ̈ʎI·ă .Σ !ffŜyˊ{Σ 5I·ǿõI·NyˊΣ 5Σ /KI·NNdzI· !Σ /NdzÎ CΣ /NdzÎ /5 óнлмоύ .NJI·ʎyˊ-ŘŜNΩ̈ØŜŘ ySdzNΩ̈2Ω̈N2LJKʎÖ ŦI·ÖΩ̈2NΩ̈Σ I·ÖΩ̈ʎyƎ I·Ω̈ Ω̈KŜ ăLJʎyI·f Ö2NΩ̈Ř fŜØŜfΣ LJI·NΩ̈ʎÖʎLJI·Ω̈ŜăʎyˊõfI·ŘŘŜN KèLJŜNI·ÖΩ̈ʎØʎΩ̈è I·yŘ NΩ̈ŜŦŜNΩ̈ŜŘ LJI·ʎyˊ ŘdzNΩ̈ʎyƎ ÖKN2yʎÖ õfI·ŘŘŜN ʎyŦfI·YˊYˊI·Ω̈ʎ2y㊠ bŜdzN2ăÖʎŜyÖŜ нопʏуу-млн㊠

DI·õŜffI· DΣ .ŜN3ƎNΩ̈Syˊ¢Σ ¦ØŜfΩdză . óмффнύ I ěLJŜNΩ̈Ω̈2LJKè I·yŘ NΩ̈ŜØŜNΩ̈ăI·f 2Ŧ KèLJŜNΩ̈Ω̈N2LJKè ʎyˊ NJI·Ω̈ LJŜfØʎÖ ƎI·yƎfΩ̈2yˊ ySdzN2yã㊠ W bŜdzN2ÖěΩ̈2f нмʏспф-ссн㊠

DI·ØI·ÎÎʎ LΣ YdzYˊI·N w5Σ aÖaI·K2yˊ{.Σ /2KŜyˊW óмфффύ DN2ǿΩ̈K NΩ̈ŜăLJ2yãŜă 2Ŧ ŘʎŦŦŜNΩ̈ŜyΩ̈ ădzõLJ2LJdzfI·Ω̈ʎ2yã 2Ŧ I·ŔdzfΩ̈ ăŜyã2Nè yŜdzN2yã Ω̈2 ySdzN2Ω̈N2LJKʎÖ ŦI·ÖΩ̈2Nă ʎyˊ ØʎΩ̈N2㊠ ¢KŜ 9dzN2LJŜI·yˊ ƨ2dzNyI·f 2Ŧ ySdzN2ăÖʎŜyÖŜ ммʏоплр-опмп㊠

W2KyãyˊD[Σ [I·LJI·ŘI·Ω̈ w óнллнύ aʎΩ̈ƎŜyˊI·ÖΩ̈ʎØI·Ω̈ŜŘ LJN2Ω̈ŜʎyˊŁʎyI·ăŜ LJI·Ω̈Kǿl·èă YˊŜŘʎI·Ω̈ŜŘ õè 9wYΣ WbYΣ I·yŘ LJoy LJN2Ω̈ŜʎyˊŁʎyI·ăŜă㊠ {ÖʎŜyÖŜ нфуʏмфмм-мфмн㊠

YŜNΩ̈ .WΣ .NJI·Rõdz N̈è 9WΣ .ŜyƳΩ̈Ω̈ 5[Σ ¢NΩ̈ØŜŘʎ taΣ 5I·ăăI·yˊ tΣ CNΩ̈ŜyÕK WΣ {KŜfΩ̈2yˊ 5.Σ aÖaI·K2yˊ{.Σ ¢K2YˊLJă2yˊ{² óмфффύ .NJI·ʎyˊ-ŘŜNΩ̈ØŜŘ yŜdzN2Ω̈N2LJKʎÖ ŦI·ÖΩ̈2N YˊŘdzfI·Ω̈ŜăyƨÖʎÖŜLJΩ̈ÁØŜ ăŜyã2NΩ̈è ʎyLJdzΩ̈ă I·yŘ baŜ!-ŜØ2Ł ŜŘ NΩ̈ŜăLJ2yãŜă ʎyˊΩ̈KŜ NJI·Ω̈ ăLJʎyI·f Ö2NΩ̈Ř㊠ W bŜdzN2-ăÖʎ мфʏрмоу-рмпу㊠

YʎYˊLJʎyã]ʎ YΣ WŜfʎyã]ʎ {Σ aŜI·N2ǿ Y óмфффύ ¢KŜ I·yΩ̈ʎ-LJтр I·yΩ̈ʎ-õ2ŘèΣ a/мфнΣ I·yŘ õNJI·ʎyˊ-ŘŜNΩ̈ØŜŘ yŜdzN2Ω̈N2LJKʎÖ ŦI·ÖΩ̈2N ʎyKʎõʎΩ̈ yŜNΩ̈ØŜ ƎN2ǿΩ̈K ŦI·ÖΩ̈2N-ŘŜLJŜyŘŜyΩ̈ yŜdzNΩ̈ʎÖŜ ƎN2ǿΩ̈K FN2Yˊ I·ŔdzfΩ̈ ăŜyã2NΩ̈è yŜdzN2yã㊠ bŜdz-N2ăÖʎŜyÖŜ фоʏнро-нсо㊠

YNΩ̈ŜyÎ bwΣ ²ŜI·ØŜN [/ óмффуύ {LJN2dzΩ̈ʎyƎ 2Ŧ LJNΩ̈ʎYˊI·NΩ̈è I·ŦŦŜNΩ̈ŜyΩ̈ ŦʎõŜNΩ̈ă I·ŦΩ̈ŜN ăLJʎyI·f Ö2NΩ̈Ř ΩNJI·yãŜÖΩ̈2yˊ ʎyˊ Ω̈KŜ NJI·Ω̈㊠ bŜdzN2ăÖʎŜyÖŜ урʏппо-пру㊠

YNΩ̈ŜyÎ bwΣ aŜI·]ʎyˊ{hΣ YNJI·ăăʎ2dz]2Ø !±Σ ²ŜI·ØŜN [/ óмфффύ bŜdzΩ̈NJI·ŦʎÎʎyƎ ʎyΩ̈NJI·ăLJʎyI·f ySNØŜ ƎN2ǿΩ̈K ŦI·ÖΩ̈2N õf2Ö]ă I·dzΩ̈2y2YˊʎÖ ŘèăNΩ̈ŜŦfŜÈʎI· ÖI·dzăŜŘ õè ăLJʎyI·f Ö2NΩ̈Ř ʎyƨdzNΩ̈è㊠ W bŜdzN2ăÖʎ мфʏтплр-тпмп㊠

YNΩ̈ăKyI· aΣ bI·NJI·yƎ I óнллуύ ¢KŜ Ö2YˊLJfŜÈʎΩ̈è 2Ŧ YˊʎΩ̈2ƎŜyˊ-I·ÖΩ̈ʎØI·Ω̈ŜŘ LJN2Ω̈ŜʎyˊŁʎyI·ăŜă óa!tYăΩ̈ YˊI·ŘŜ ăʎYˊLJfŜ㊠ /ŜffdzfI·N I·yŘ YˊŦfŜÖdzfI·N fŦŦŜ ăÖʎŜyÖŜă Y /a[{ срʏорнр-орпп㊠

YdzWI óнллсύ ¢KŜ YˊI·yI·ƎŜYˊŜyΩ̈ 2Ŧ ySdzN2ƎŜyʎÖ õfI·ŘŘŜN I·yŘ Ijdz I·fΩ̈è 2Ŧ fŦŜ ʎyˊăLJʎyI·f Ö2NΩ̈Ř ʎyƨdzNΩ̈è㊠ .W¦ LyΩ̈ фуʏтоф-тпр㊠

[ŜØŜ₦ LΣ /dzyÝƛyƎK·Y WΣ D₦ƛãú WΣ ¸ƛLJ tYΣ a·fÔ·yƎƛ2 a óнллоl·ú wŜfŜ·ãŜ 2Ŧ . 5bC ·yŘ D! . ! ƛyˇúKŜ Ř2₦-ãl·f K2₦yˇ 2Ŧ ySdz₦2LJl·úKƛÕ ₦·úãΦ ¢KŜ 9dz₦2LJŜ·yˇ ŝ2dz₦y1·f 2Ŧ ySdz₦2ãÕƛŜyÕŜ муÝммсф-ммтпΦ

[ŜØŜ₦ LW΢ tŜÎŜú {Σ aÕa·K2yˇ{ . Σ a·fÔ·yƎƛ2 a óнллоõú ¢KŜ ãƛƎyl·fƛyƎ Õ2YLJ2ySyũã 2Ŧ ãŜyã2₦Wè ŦƛõŜ₦ ú₦·yãY·ƛããƛ2yˇ ƛyØ2ŧfØŜŘ ƛyˇúKŜ ·ÕúƛØl·úƛ2yˇ 2Ŧ 9wY a ! t]ƛyl·ãŜ ƛyˇúKŜ Y˙2dzãŜ Ř2₦ãl·f K2₦yΦ a2fŜÕdz-fl·₦ ·yŘ ÕŜffdzfl·₦ ySdz₦2ãÕƛŜyÕŜã нпÝнрф-нтлΦ

aŜ₦ƎKƛ !Σ /·₦Y·ƛƎyƩú2 DΣ D2õõ2 {Σ [2ããƛ [Σ {l·fƛ2 /Σ ±Ŝ₦Ǝyl·y2 ! aΣ ½2yúl· a óнллпú bŜdz₦2ú₦2LJKƛyã ƛyˇ ãLJƛyl·f Õ2₦Ř y2ÕƛÕŜLJúƛØŜ LJl·úKØl·èãΦ t₦2Ǝ₦Ŝãã ƛyˇõ₦·ƛyˇ₦ŜãŜl·₦ÕK мпсÝнфм-онмΦ

aŜ₦ƎKƛ !Σ {l·fƛ2 /Σ DK₦₦ƛ !Σ [2ããƛ [Σ CŜ₦₦ƛyƛ CΣ . ŜúŜffƛ /Σ . ·₦Ř2yƛ w óнллуú .5bC ·ã · LJl·ƛyˇ Y˙2Řdzfl·úŕ2₦Φ t₦2Ǝ₦Ŝãã ƛyˇyŜdz₦2õƛ2f2Ǝè урÝнфт-омтΦ

aƛèl·Îl·úŕ2 aΣ {l·ãl·úŕ2Yƛ YΣ I ƛ₦l·Ǝl·úl· {Σ {dzƎl·èl· YΣ /Kl·yÕŜf-f2₦ a .Σ ŘŜ D₦2l·ú ² /Σ ¸2ãKƛYdz₦l· b óнллуú {dzLJ-LJ₦Ŝããƛ2yˇ 2Ŧ ŘŜú₦dzã2₦-ãLJKƛyÕúŜ₦ Řèäèy Ŝ₦Ǝƛl· õè D! . !-₦ŜÕŜLJú2₦ l·ÕúƛØl·úƛ2yˇ ƛyˇ úKŜ fdzYõ2ãl·Õ₦l·f ãLJƛyl·f Õ2₦Ř ƛyˇ ãLJƛyl·f Õ2₦Ř-ƛyŕdz₦ŜŘ ₦l·úãΦ !Y W tKèãƛ2f wŜƎdzf LyúŜƎ₦ /2YLJ tKèãƛ2f нфрÝwooc-опнΦ

aƛèl·Îl·úŕ2 aΣ {dzƎl·èl· YΣ D2ƛyã ² CΣ ² 2ffŜ 5Σ D2ãä Ww΢ /Kl·yÕŜff2₦ a .Σ ŘŜ D₦2l·ú ² /Σ Df2₦ƛ2ã2 W/Σ ¸2-ãKƛYdz₦l· b óнллфú l Ŝ₦LJŜã ãƛYˇLJfŜÈ Ø₦dzã ØŜÕú2₦-YˇŜŘƛl·úŜŘ ƎŜySŜ ŘŜfƛØŜ₦è 2Ŧ Ǝfdzúl·YˇlÕ l·ÕƛŘ ŘŜÕl·₦-õ2ÈèfI·ãŜ ₦ŜŘdzÕŜã ŘŜú₦dzã2₦ 2ØŜ₦l·ÕúƛØƛúè ƛyˇ ãLJƛ-yl·f Õ2₦Ř-ƛyŕdz₦ŜŘ ₦l·úãΦ DŜySŜ ¢KŜ₦ мсÝссл-ссуΦ

bl·èf2₦ w[Σ w2õŜ₦úã2yˇ !D΢ !ffŜyˇ {W΢ {Šããƛ2yã w.Σ /fl·₦ŜΣ !w΢ al·ã2yˇ DDΣ .dz₦ãú2yˇ WW΢ ¢èfŜ₦ {W΢ ² ƛfÕ2Õ] DYΣ 5l·øõl·₦yˇ 5 óнллнú ! ŘƛãÕ₦ŜúŜ Ř2Yl·ƛyˇ 2Ŧ úKŜ KdzYl·yˇ ¢₦] .₦ŜÕŜLJú2₦ ŘŜŦƛySã úKŜ õƛyŘƛyƎ ãƛúŜã Ŧ2₦ .5bC ·yŘ b¢-п .ƛ2ÕKŜY .ƛ2LJKèã wŜã /2YYdzyˇнфмÝрлм-рлтΦ

hfƛØl· ! !Σ W₦ØΣ !ú]ƛyã / aΣ /2LJŜyl·ƎfŜ [Σ .l·y]Ŝ₦ D! óнллсú !ÕúƛØl·úŜŘ Õ-Wdzyˇ b-úŜ₦YƛYl·f]ƛyl·ãŜ ãã ₦ŜIjdz₦ŜŘ Ŧ2₦ l·È2yˇŦ2₦YˇI·úƛ2y Φ W bŜdz₦2ãÕƛ нсЧфпсн-фптлΦ

hyŘI·₦Îl· ! .Σ ¸Ŝ ½Σ IdzfãŜõ2ãÕK /9 óнллоú 5ƛ₦ŜÕú ŜØƛ-ŘŜyÕŜ 2Ŧ LJ₦ƛYl·Wè l·ŦŦŜ₦Ŝyú ãLJ₦2dzúƛyƎ ƛyˇ Řƛãúl·yú ãŜƎYŜyúã Ŧ2ff2ØƛyƎ ãLJƛyl·f Õ2₦Ř ƛyˇdz₦Wè ƛyˇ úKŜ ₦l·úY Õ2f2Õl·fƛÎl·úƛ2yˇ 2Ŧ D!t-по l·yŘ /Dwt Φ 9ÈLJ bŜdz₦2f мупÝото-оулΦ

tŜÎŜú {Σ aÕa·K2yˇ{ . óнллсú bŜdz₦2ú₦2LJKƛyãY YˇŜŘƛl·úŕ2₦ã l·yŘ Yˇ2Řdzfl·úŕ2₦ã 2Ŧ LJl·ƛyÕ !yÝdz wŜØ bŜdz₦2ãÕƛ нфЧрлт-роуΦ

tŜÎŜú {Σ /dzyÝƛyƎK·Y WΣ tl·úŜf WΣ D₦ƛãú WΣ Dl·Øl·ÎÎƛ LΣ [ŜØŜ₦ LW΢ al·fÔl·yƎƛ2 a óнллнl·ú .5bC Yˇ2Řdzfl·úŜã ãŜyˇ-ã2₦Wè ySdz₦2yˇ ãèyl·LJúƛÕ l·ÕúƛØƛúè õè l· Ŧl·Õƛfƛúŕ·úƛ2yˇ 2Ŧ D! . ! úƛ₦l·yãYƛããƛ2yˇ ƛyˇúKŜ Ř2₦ãl·f K2₦yΦ a2f /Ŝff bŜdz₦2ãÕƛ нмÝрм-снΦ

tŜÎŜú {Σ al·fÔl·yƎƛ2 aΣ [ŜØŜ₦ LW΢ tŜ₦]ƛyú2yˇ a{Σ ¢K2YLJ-ã2yˇ {² Σ ² ƛffƛl·Yˇã wW΢ aÕal·K2yˇ{ . óнллнõú b2ÈƛŁdzã ãúƛYˇdzfl·úŕ2yˇ ƛyŘdzÕŜã ¢₦] ₦ŜÕŜLJú2₦ l·yŘ Ř2óyãú₦Ŝl·Y 9wY LJK2ãLJK2₦èfl·úŕ2yˇ ƛyˇ ãLJƛyl·f Ř2₦ãl·f K2₦yΦ a2fŜÕdzfl·₦ l·yŘ ÕŜffdzfl·₦ ySdz₦2ãÕƛ-ŜyÕŜã нмÝсуп-сфрΦ

wl·õÕKŜØã]è !D óнллсú {ŜƎYŜyúl·f 2₦Ǝl·yÎl·úŕ2yˇ 2Ŧ ãLJƛyl·f ₦ŜŦfŜÈŜã YˇŜŘƛl·úŕyƎ l·dzú2y2YˇlÕ ŘèãƛŜŦfŜÈƛl· l·Ŧú Ŝ₦ ãLJƛyl·f Õ2₦Ř ƛyˇdz₦èΦ t₦2Ǝ . ₦l·ƛyˇwŜã мрнÝнср-нтпΦ

wl·YŜ₦ [aΣ aÕtKl·ƛf [¢Σ .2₦ãã2ŦŦ WCΣ {2₦ƛf [W΢ Yl·l·yˇ ¢YΣ [ŜŜ W l Σ {l·dzyŘŜ₦ã W²Σ l ø ƛ [tΣ wl·YŜ₦ a{ óнллтú 9yˇ-Ř2ŦŜyŸ2dzã ¢₦] . fƛƎl·yŘã ãdzLJLJ₦Ŝãã Ŧdzyŏúƛ2yl·f YŜÕK-l·y2ãŜyã2₦Wè LJfl·ãúƛÕƛúè ƛyˇ úKŜ ŘŜl·ŦŦŜ₦ŜyúŜŘ ãLJƛyl·f Õ2₦Ř Φ W bŜdz₦2ãÕƛ нтЧрумн-руннΦ

{l·fƛ2 /Σ !ØŜ₦ƛff {Σ t₦ƛŜãúfŜè W±Σ aŜ₦ƛƎKƛ ! óнллтú /2ãú2₦l·ƎŜ 2Ŧ .5bC ·yŘ ySdz₦2LJŜLJúƛŘŜã øƛúKƛyˇ ƛyŘƛØƛŘdz·f ŘŜyãŜ-Õ2₦Ŝ ØŜãƛÕfŜã ƛyˇÕŜyúƛl·f l·yŘ LJŜ₦ãLJKŜ₦l·f ySdz-₦2yãΦ 5ŜØŜf2LJYˇŜyúl·f ySdz₦2õƛ2f2Ǝè стЧонс-ооуΦ

{l·ãl·]ƛ YΣ /Kl·yÕŜff2₦ a .Σ tKŜfl·yˇ a²Σ ¸2]2èl·Yl· ¢Σ C₦l·ãŜ₦ ahΣ {Ŝ]ƛ {Σ Ydzõ2 YΣ YdzY˙2yˇ I Σ D₦2l·ú ² /Σ ¸2ãKƛ-Y˙dz₦l· b óнллнú 5ƛl·õŜúƛÕ Õèãú2LJl·úKè Õ2₦₦ŜfI·úŜã øƛúK l· f2yƎ-úŜ₦Y ŘŜÕ₦Ŝl·ãŜ ƛyˇ ySdz₦ØŜ Ǝ₦2øúK Ŧl·Õú2₦ fŜØŜfã ƛyˇúKŜ õfl·ŘŘŜ₦ l·yŘ fdzYõ2ãl·Õ₦l·f Ř2₦ãl·f ₦2ƩúDl·yƎfƛl·Φ W ¦₦2f мсуЧмнрф-мнспΦ

{Õ₦Yˇúƛ] {YΣ I ²2₦₦úK WW΢ W2ŜYl·ƛ waΣ ²ƛèyúƩŜã wΣ 9ƛ2ƎŜyƩ₦l·l·Y {Σ ŘŜ .₦dzƛ2yˇ tΣ DŜ2₦₦ƎƛŁdz /Σ ŘŜ W2yƎ ! tΣ Øl·yˇ h2èŜyˇ !Σ ±Ŝ₦Kl·ƎŜ aΣ /2₦yŜfƛããŜ [bΣ ¢22ySyˇ wCΣ ±ŜfŘ-]l·Y˙LJ ² W óнлммú !dzúƎ2Yl·úŜŘ l·yl·fèãƛã 2Ŧ ySdz₦2yl·f Y˙2₦LJK2f2Ǝèΐ ãèyl·LJãŜ ydzYõŜ₦ l·yŘ ãèyl·LJúƛÕ ₦Ŝ-Õ₦dzƛúYˇŜyúΦ W2dz₦yl·f 2Ŧ ySdz₦2ãÕƛŜyÕŜ Y˙ŜúK2Řã мфрÝмур-мфоΦ

{Ŝ]ƛ {Σ {l·ãl·]ƛ YΣ Ƚ3l·øl· ¸Σ bƛãKƛÎl·øl· hΣ /Kl·yÕŜff2₦ a .Σ 5Ŝ D₦2l·ú ² /Σ ¸2ãKƛYdz₦l· b óнллпú {dzLJLJ₦ŜããƛyˇŸ2Ŧ ŘŜú₦dzã2₦-ãLJKƛyÕúŜ₦ Řèãäèy Ŝ₦ƎƛI· õè ƛYˇYdzy2ySdz-úŃl·fƛÎl·úƛ2yˇ 2Ŧ ySØŜ Ǝ₦2øúK Ŧl·Õú2₦ ƛyˇ fdzYõ2ãl·Õ₦l·f ãLJƛyl·f Õ2₦Ř ƛyˇ ãLJƛyl·f Õ2₦Ř ƛyŕdz₦ŜŘ ₦l·úãΦ W ¦₦2f мтмЧпту-пунΦ

{Ŝ]ƛ {Σ {l·ãl·]ƛ YΣ C₦l·ãŜ₦ ahΣ Ƚ3l·øl· ¸Σ bƛãKƛÎl·øl· hΣ /Kl·y-ÕŜff2₦ a .Σ ŘŜ D₦2l·ú ² /Σ ¸2ãKƛYdz₦l· b óнллнú LY-Ydzy2ySdz₦l·fƛÎl·úƛ2yˇ 2Ŧ ySØŜ Ǝ₦2øúK Ŧl·Õú2₦ ƛyˇ fdzYõ2ãl·Õ₦l·f ãLJƛyl·f Õ2₦Ř ƛyŕdz₦ŜŘ ₦l·úãΦ W ¦₦2f мсуЧннсф-ннтпΦ

{ƛYˇLJãã2yˇ [!Σ 9yƎ WW΢ IãƛŜK W¢Σ ²2ffŜ 5[óнлмнú ¢KŜ KŜl·fúK l·yŘ fƛfŜ LJ₦ƛ2₦₦úƛŜã 2Ŧ ƛyŘƛØƛŘdz·fã øƛúK ãLJƛyl·f Õ2₦Ř ƛyŕdz₦ŜŘY l· ãèãúŜYl·úƛÕ ₦ŜØƛŜøΦ W bŜdz₦2úŃl·dzYl· нфЧрпу-мрррΦ

{úŜŜ₦ã ²5Σ /₦ŜŜŘ2yˇ 5W΢ ¢dzúúfŜ W. óмффсú LYˇYdzyƛúè ú2 ySØŜ Ǝ₦2øúK Ŧl·Õú2₦ LJ₦ŜØŜyúã l·ŦŦŜ₦Ŝyú LJfl·ãúƛÕƛúè Ŧ2ff2øƛyƎ dz₦ƛyl·₦è õfl·ŘŘŜ₦ KèLJŜ₦ú₦2LJKè W ¦₦2f мррЧотф-оурΦ

{úŜŜ₦ã ²5Σ Y2fõŜÕ] {Σ /₦ŜŜŘ2yˇ 5Σ ¢dzúúfŜ W. óмффмú bŜØŜ Ǝ₦2øúK Ŧl·Õú2₦ ƛyˇúKŜ dz₦ƛyl·₦è õfl·ŘŘŜ₦ 2Ŧ úKŜ l·Řdztú ₦ŜƎdzfl·úŜã yŜdz₦2yl·f Ŧ2₦Yˇ l·yŘ ŦdzyÕúƛ2yΦ W /fƛyˇ LyÕŜãú yyЧмтлф-мтмрΦ

¢K2YLJã2yˇ{²Σ .ŜyŸŜúú 5[Σ YŜ₦₦ .W΢ .₦l·Řõdz₦è 9W΢ aÕal·K2yˇ{ . óмфффú .₦l·ƛyˇ-ŘŜ₦ØŜŘ yŜdz₦2ú₦2LJKƛÕ Ŧl·Õú2₦ ƛã l·yˇ ŜyŘ2ƎŜy2dzã Y˙2Řdzfl·ú2₦ 2Ŧ yˇ2ÕƛÕŜLJúƛØŜ ₦Ŝ-ãLJ2yãŜã ƛyˇ úKŜ ãLJƛyl·f Õ2₦ŘΦ t₦2ÕŜŜŘƛyƎã 2Ŧ úKŜ bl·úƛ2yl·f !Õl·ŘŜYè 2Ŧ {ÕƛŜyÕŜã 2Ŧ úKŜ ¦yúŜŘ {úl·úŜã 2Ŧ !YŜ₦ØÌ· фсЧттмп-ттмуΦ

¢dzúúfŜ W.Σ {úŜŜ₦ã ²5 óмффнú bŜ₦ØŜ Ǝ₦2øúK Ŧl·Õú2₦ ₦ŜãLJ2yˇ ãƛØŜyŜãã 2Ŧ Õdzfúdz₦ŜŘ Yl·2₦ LJŜfØƛÕ Ǝl·yƎfƛ2yˇ ySdz-₦2yã Ŧ₦2Yˇ úKŜ l·Řdztú ₦l·ú . ₦l·ƛyˇ ₦ŜãŜl·₦ÕK рууЧнф-плΦ

±ƛÎÎl·₦Ř a! óмфффú !fúŜ₦l·úƛ2yã ƛyˇ Ǝ₦2øúK-l·ãã2Õƛl·úŜŘ LJ₦2-úŜƛyˇóD! t-поú ŜÈLJ₦Ŝããƛyˇƛyˇf2øŜ₦ dz₦ƛyl·₦è úl·Õú

LJI·ű K ø I·èă Ŧ2ff2 ø ʎyƎ ŎK₦2yʎŎ ăLJʎyƬ·f Ŏ2₦Ř ʎy¢dz₦è¢ {2Y I·ú2ăŜyã a2ű wŜă мсʸoсф-оум¢

±ʎÎÎI·₦Ř a ! óнллсú bŜdz₦2ŐKŜY ʎŎI·f LJfI·ăűʎŎʎűè I·yŘ úKŜ ₦2fŜ 2Ŧ ySdz₦2ú₦2LJKʎŎ ŦI·Ŏű2₦ă ʎy ōfI·ŘŘŜ₦ ₦ŜŦfŜÈ LJI·úK-ø I·ěă I·ŦűŜ₦ ăLJʎyƬ·f Ŏ2₦Ř ʎy¢dz₦è¢ t₦2Ǝ . ₦I·ʎy wŜă мрнʸфт-ммр¢

² ŜI·ØŜ₦ [/Σ ±Ŝ₦ƎKŜăŜ tΣ . ₦dzŐŜ W /Σ CŜKfʎyƎă a DΣ Y₦ŜyÎ bwΣ a I·₦ăK 5w óнллмú ! dzű2y2YʎŐ Řèǎ₦ŜŦfŜÈʎI· I·yŘ LJ₦ʎY I·₦è I·ŦŦŜ₦Ŝyű ăLJ₦2dzűʎyƎ I·ŦűŜ₦ ŎfŁLJ-Ŏ2YLJ₦Ŝăǎʎyʎydz₦è 2Ŧ úKŜ ₦I·ű ăLJʎyƬ·f Ŏ2₦Ř¢ W bŜdz-₦2ú₦I·dzY I· муʸммлт-мммф¢

² ʎŘY I·yy /Σ Dʎōǎ2y {Σ W I·₦LJŜ a . Σ W2Kyã2y D[óмфффú a ʎű2-ƎŜy-I·ŎűʎØI·úŜŘ LJ₦2úŜʎy ʃʎyƬ·ăŜʏ Ŏ2yãŜ₦ØI·úʎ2y 2Ŧ I· úK₦ŜŜ-ʃʎyƬ·ăŜ Y2ŘdzfŜ Ŧ₦2Y èŜI·ăű ű2 KdzY I·y¢ tKèǎʎ2f2ƎʎŐI·f ₦ŜØʎŜøă тфʸмпо-мул¢

½ʎYYŜ₦Y I·yy a óмфуoú 9űKʎŐI·f ƎdzʎŘŜfʎyŜă Ŧ2₦ ʎyØŜăúʎƎI·-úʎ2yã 2Ŧ ŜÈLJŜ₦ʎYŜyűI·f LJI·ʎy ʎy Ŏ2yãŎʎ2dzǎ I·yʎY I·fã¢ t I·ʎy мсʸмлф-ммл¢

½ʎyŐʃ b5Σ wI·ŦdzǎŜ ±CΣ 52øyʎŜ W ² óнллтú {LJ₦2dzűʎyƎ 2Ŧ /Dwt LJ₦ʎY I·₦è I·ŦŦŜ₦Ŝyűă ʎy fdzY ō2ǎI·Ŏ₦I·f ăLJʎyƬ·f Ŏ2₦Ř LJ₦ŜŐŜŘŜă ŜYŜ₦ƎSyŐŜ 2Ŧ ōfI·ŘŘŜ₦ I·ŐűʎØʎúè I·ŦűŜ₦ ăLJʎyƬ·f ʎy¢dz₦è¢ 9ÈLJ bŜdz₦2f нлпʸттт-тфл¢

Publication V

Transient receptor potential vanilloid 1 mediates nerve growth factor-induced bladder hyperactivity ad noxious input

Bárbara Frias, Ana Charrua, António Avelino, Martin C. Michel, Francisco Cruz, Célia D. Cruz

British Journal of Urology International (2012) 110:422-8

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Transient receptor potential vanilloid 1 mediates nerve growth factor–induced bladder hyperactivity and noxious input

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Accepted for publication 8 February 2012

OBJECTIVES

- To explore the role of transient receptor potential vanilloid 1 (TRPV1) in the excitatory effects of chronic administration of nerve growth factor (NGF) on bladder-generated sensory input and reflex activity.
- To explore new therapeutic targets for bladder dysfunction.

MATERIALS AND METHODS

- Wild-type (WT) and TRPV1 knockout (KO) mice received daily intraperitoneal injections of NGF (1 μg/10 g) or saline for a period of 4 days, during which time thermal sensitivity was evaluated daily. On the 5th day, mice were anaesthetized and cystometries were performed. The frequency, amplitude and area under the curve (AUC) of bladder reflex contractions were determined.
- c-Fos expression was evaluated on L6 spinal cord sections of WT and TRPV1 KO mice treated with saline or chronic NGF by immunohistochemistry.
- TrkA receptor staining intensity was determined in L6 spinal cord sections and respective dorsal root ganglia of WT and TRPV1 KO mice.

What's known on the subject? and What does the study add?
The interaction between the TRPV1 and NGF systems has been addressed only in the context of acute somatic pain. The present study expands this view and indicates that this interaction remains operative and is important as a mechanism for chronic visceral pain and dysfunction. Moreover, it further stresses the need to develop more specific and effective TRPV1 antagonists for clinical use.

RESULTS

- Repeated administration of NGF induced thermal hypersensitivity in WT but not in TRPV1 KO mice.
- The frequency of bladder contractions of saline-treated WT and TRPV1 KO mice was similar, the values respectively being 0.45 ± 0.12/min and 0.46 ± 0.16/min. Treatment with NGF enhanced bladder reflex activity in WT mice to 1.23 ± 0.41/min ($P < 0.05$). In NGF-treated KO mice, the frequency of bladder contractions was 0.60 ± 0.05/min. Irrespective of treatment, no differences were observed in the amplitude of bladder contractions of WT and TRPV1 KO mice. The AUC was significantly increased in NGF-treated WT-mice, when compared with saline-treated WT-mice. No changes were found in AUC of saline-treated and NGF-treated TRPV1 KO mice.
- Chronic administration of NGF resulted in a significant increase of spinal c-Fos expression in WT mice ($P < 0.05$ vs KO animals), but not in TRPV1 KO animals.
- TrkA expression was similar in WT and TRPV1 KO mice.

CONCLUSIONS

- NGF-induced bladder overactivity and noxious input depend on the interaction of NGF with TRPV1.
- The lack of bladder overactivity in TRPV1 KO mice treated with NGF does not represent loss of TrkA expression.
- TRPV1 is essential for NGF-driven bladder dysfunction and represents a bottleneck target in bladder pathologies associated with NGF up-regulation.

KEYWORDS

nerve growth factor, transient receptor potential vanilloid 1, bladder overactivity

INTRODUCTION

Transient receptor potential vanilloid (TRPV1) is a transmembrane ion channel. It is not essential for physiological bladder function in healthy animals as TRPV1 knockout (KO) mice exhibit normal or near-normal bladder activity [1]. In contrast, TRPV1 is essential for detrusor overactivity and increased voiding frequency accompanying acute and chronic cystitis in rodents [1]. In overactive bladder syndrome and interstitial cystitis/bladder pain syndrome TRPV1 overexpression has been described in the bladder wall, both in urothelial cells and in

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suburothelial nerve fibres [2,3]. The expression of neuronal TRPV1 correlates with the intensity of bladder pain [2] whereas the intensity of TRPV1 in the urothelium of patients with sensory urgency inversely correlates with the volume of urine associated with the first desire to void [4].

Nerve growth factor (NGF) is a tissue-derived neurotrophin essential for the survival and differentiation of sensory neurons [5], which acts through the tyrosine kinase receptor TrkA [6]. It is acutely released after an inflammatory insult and significantly contributes to the swift modification of the pain threshold and visceral activity [7]. NGF has also been considered to be sufficient to elicit cutaneous hyperalgesia [8]. Exogenous administration of NGF induces detrusor overactivity in naive rats [9,10], as well as up-regulating the expression of spinal c-Fos and the firing of bladder nociceptive fibres [9,11], in agreement with the strong expression of TrkA receptors by bladder sensory afferents [6].

The identification of downstream effectors of NGF-induced effects has only recently become a focus of systematic research. In this context, TRPV1 was found to be essential for the development of thermal hyperalgesia after acute administration of NGF [12]. In mice that received intraplantar NGF, the latency of paw withdrawal to a noxious thermal stimulus, an indication of thermal hyperalgesia, was substantially decreased in wild-type (WT) mice but not in TRPV1 KO mice [12]. In this study we evaluated if the interplay between NGF and TRPV1, important for somatic pain, is also present in NGF-mediated bladder overactivity and noxious input.

MATERIALS AND METHODS

TRPV1 KO female mice from The Jackson Laboratory (Bar Harbor, ME, USA) and WT female mice belonging to the same strain (C57BL/6; $n = 6$/group) from the Instituto de Biologia Molecular e Celular colony (Porto, Portugal) with an average weight of 25 g were used. Animals were maintained in the animal house at 22 °C and 60% humidity under a 12-h light/dark cycle. All experiments were carried out according to the European Commission Directive of 22 September 2010 (2010/63/EU) and the ethical guidelines for investigation of experimental pain in animals [13]. All efforts were made to reduce the number of animals used.

The NGF was purchased from Promega (Madison, WI, USA). The antibody against TrkA receptor, made in rabbit, was purchased from Millipore, Watford, UK. The antibody against c-Fos, made in rabbit, came from Millipore, Watford, UK. Biotin-conjugated swine anti-rabbit antibody came from Dakopatts A/5 (Copenhagen, Denmark). The ABC Vectastain Elite kit (ABC, avidin–biotin complex) and the conjugate horseradish peroxidase were purchased from Vector Laboratories (Peterborough, UK). Antibodies and the ABC complex were prepared in PBS 0.1 M containing 0.3% Triton X-100 (PBST). For cystometry and terminal handling, mice received a subcutaneous bolus of urethane (1.2 g/kg) as anaesthetic.

The NGF was given as a daily intraperitoneal injection (1 μg/10 g) for 4 days. The dose of NGF was chosen according to previous studies [14]. Sterile saline was given as a control. In addition, because NGF is known to induce thermal hyperalgesia in WT animals [12], the effects of repeated NGF administration on thermal sensitivity were evaluated daily (see below) to assure the biological action of NGF. On day 5, mice were anaesthetized and cystometry was performed, after which animals were perfusion-fixed and the L6 spinal cord segment was collected for c-Fos assessment.

The hot-plate test was used to measure the response latencies to thermal noxious stimuli. Hence, NGF-injected TRPV1 KO and WT mice were placed in individual chambers (10 × 20 × 14 cm) and allowed to acclimate for 5 min. Latencies were determined before and 4 h after each NGF injection. For that, animals were placed on the hot-plate apparatus (Series 8, model PE34, IITC Life Sciences, Woodland Hills, CA, USA). The platform was maintained at 35.0 ± 0.1 °C and temperature was increased up to 52.5 °C. The time spent between placement of the animal on the platform and positive behavioural responses (jumping on the platform and/or licking of the hindpaws) was registered as the response latency.

To assess bladder function, cystometry was performed in TRPV1 KO and WT mice treated with saline or NGF on day 5 ($n = 6$).

Anaesthesia was induced by a subcutaneous injection of urethane and body temperature was maintained at 37 °C with a heating pad. The urinary bladder was exposed through an incision in the lower abdomen. A 25-gauge needle was inserted in the bladder dome and the urethra remained unobstructed throughout the recording period while saline was infused at a constant rate (1.6 mL/h). The frequency, amplitude of bladder contractions and area under the curve (AUC) were then measured for a period of 90 min. In all experiments, recordings were made after a 30-min stabilization period.

After cystometry, animals were perfused through the ascending aorta with cold oxygenated calcium-free Tyrode's solution (0.12 M NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2.6H_2O$, 0.4 mM $MgSO_4.7H_2O$, 1.2 mM $NaH_2PO_4.H_2O$, 5.5 mM glucose, 26.2 mM $NaHCO_3$), followed by cold 4% paraformaldehyde. The L6 spinal cord segments were post-fixed for 4 h in the same fixative solution and cryoprotected for 24 h in 30% sucrose with 0.1% sodium azide in 0.1 M phosphate buffer. Transverse 20-μm sections from the spinal cord were cut in the freezing microtome and stored in cryoprotective solution at −20 °C until further processing. When all material was collected and cut, every second spinal section from each animal was thawed and immunoreacted against c-Fos to evaluate the expression of c-Fos. Briefly, after inhibition of endogenous peroxidase activity and thorough washes in PBS and PBST, sections were incubated in 10% normal swine serum in PBST for 2 h. Sections were then incubated for 48 h at 4 °C with a specific antibody against c-Fos (1:10 000). Subsequently, sections were washed and incubated with polyclonal swine anti-rabbit biotin-conjugated antibody (1:200). To visualize the immunoreaction, the ABC conjugated with peroxidase (1:200) method was used with 3,3′-diaminobenzidine tetrahydrochloride as chromogen (DAB; 5 min in 0.05 M Tris–HCl buffer, pH 7.4 containing 0.05% DAB and 0.003% hydrogen peroxide). Sections were mounted on gelatine-coated slides and air-dried for 12 h, cleared in xylene, mounted with *Eukitt* mounting medium and cover-slipped.

For analysis of TrkA expression the WT and TRPV1 KO mice ($n = 4$ per group) were perfusion-fixed with calcium-free Tyrode's

*FIG. 1. Thermal hyperalgesia of wild-type (WT) and transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice. TRPV1 KO mice treated with nerve growth factor (NGF; &tU25CF;) or saline (○) did not present any differences on the response latencies during the 4 days of experiment. WT mice receiving saline (□) presented similar values throughout the experiment, however, WT mice intraperitoneally injected with NGF (■) showed a significant decrease (***P < 0.001) on the response latency, compared with TRPV1 KO mice treated with NGF.*

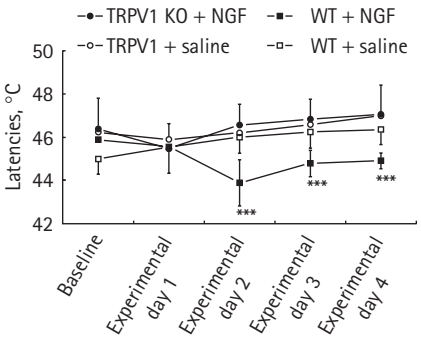
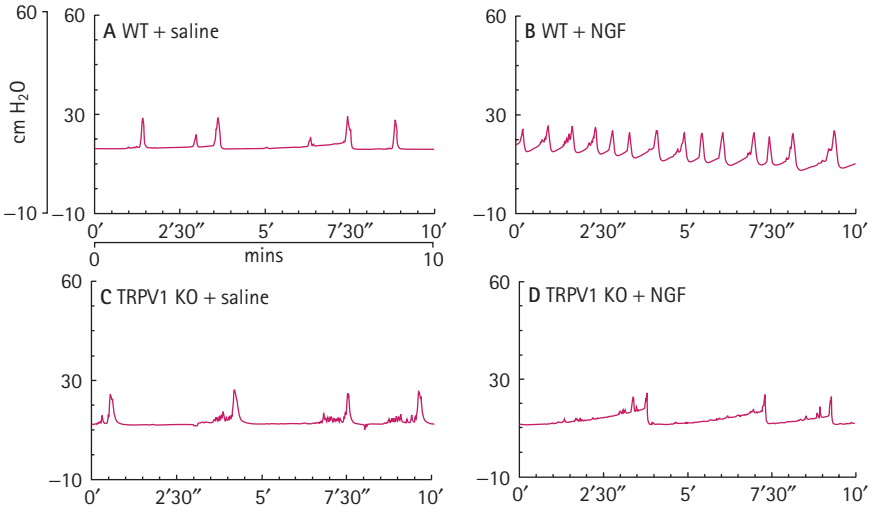


*FIG. 2. **A–D**, Representative cystometrograms of wild-type (WT) and transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice treated with saline and nerve growth factor (NGF). The frequency in WT mice receiving saline (**A**) was low but significantly increased after NGF treatment (**B**). In TRPV1 KO mice the frequency of bladder contractions was similar to that in WT mice (**C**) and was not altered by chronic NGF administration (**D**). In cystometrograms from TRPV1 KO mice receiving saline (**A**) it was possible to observed non-voiding contractions preceding urine expulsion. These contractions were slightly amplified after NGF treatment (**D**).*



solution followed by 4% paraformaldehyde. The L6 spinal cord segment and respective dorsal root ganglion (DRG) were collected. Transverse 20-μm sections of spinal cord sections were cut in the freezing microtome and stored in cryoprotective solution at −20 °C. Longitudinal 12-μm sections of L6 DRG were cut in the cryostat and stored at −20 °C. When all material was collected, sections were removed from the freezer and processed for TrkA immunoreaction, as described above for c-Fos expression. The antibody against TrkA was used at 1:1000 dilution.

Cystometrograms were evaluated using DataTrax software (Vs. 1.804; World Precision Instruments, Sarasota, FL, USA). The frequencies, amplitudes of bladder contractions and AUC were analysed using Kruskal–Wallis one-way repeated measures ANOVA. Data are presented as mean value ± SD and $P < 0.05$ was considered significant. Statistical analysis was performed with SigmaStat 3.5 software.

The number of c-Fos immunoreactive nuclei was counted in 10 non-consecutive sections from each animal and averaged. Statistical analysis was performed using ANOVA followed by the Student–Newman–Keuls post-hoc test, using the SigmaStat 3.5 software.

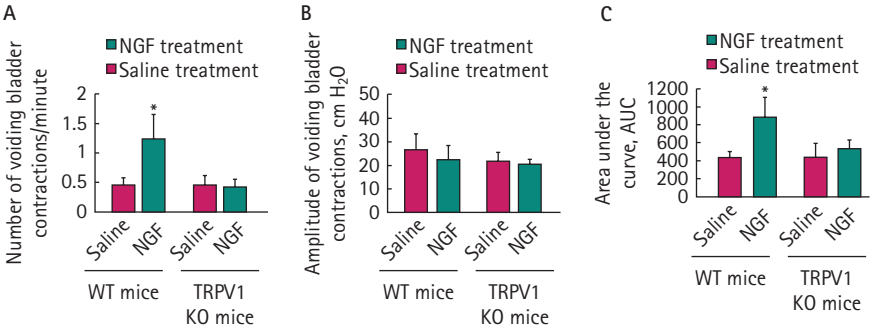
Quantification of the TrkA staining intensity was done with Fiji software (based on ImageJ, http://rsb.info.nih.gov/ij Java 1.6.0_20, 32 bit). The intensity of staining was averaged from sections per animal (DRG and spinal cord) and a reference intensity of unstained tissue was also measured by a fourth box on all sections. Background intensity was deducted from the average intensity to calculate the mean net staining intensity. The intensity of TrkA staining in WT mice treated with saline was used as control. Data were analysed by one-way ANOVA followed by the Student–Newman–Keuls post-hoc test. The data are presented as mean value ± SD and $P < 0.05$ was considered significant. Statistical analysis was carried out using the Graph Pad Prism software.

RESULTS

Daily intraperitoneal NGF injections significantly reduced thermal latency in the hot-plate test in WT mice from the day 2 onwards (Fig. 1). The baseline temperature at which WT animals presented nocifensive behaviour (jumping on the platform and/or licking of the hindpaws) was 45.9 ± 0.9 °C and was not changed by saline treatment (Fig. 1). In contrast, in WT animals the thermal latencies after each NGF injection were significantly decreased to 45.6 ± 0.7 °C, 43.9 ± 1.1 °C, 44.8 ± 0.6 °C and 44.9 ± 0.4 °C, respectively at days 1, 2, 3 and 4, respectively ($P < 0.001$ vs TRPV1 KO mice at all time points; Fig. 1). In TRPV1 KO mice the baseline latency was 46.4 ± 0.6 °C and was not changed after intraperitoneal saline injections. The temperatures registered in KO mice receiving NGF were 45.5 ± 1.1 °C, 46.6 ± 0.9 °C, 46.8 ± 0.9 °C and 47.1 ± 1.3 °C at days 1, 2, 3 and 4, respectively (Fig. 1), and were not different from baseline values.

In cystometrograms obtained from saline-treated TRPV1 KO mice, we observed non-voiding small amplitude oscillations that preceded voiding contractions (Fig. 2C). These were absent in recordings from saline-treated WT animals (Fig. 2A) and were reflected in a marginally higher AUC in KO mice (Fig. 3C), although the difference did not reach significance. The frequency of voiding contractions of WT and TRPV1 KO mice receiving saline was similar, the values respectively being 0.5 ± 0.1 and 0.5 ± 0.2

FIG. 3. ***A****, Histogram showing the mean frequency of bladder voiding contractions of wild-type (WT) and transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice treated with saline or nerve growth factor (NGF). Mice were treated with intraperitoneal injections of saline or NGF during four experimental days. At day 5, animals were anaesthetized for cystometry. The frequency of bladder contractions was only significantly increased in NGF-treated WT when compared with saline-treated WT mice (*P < 0.05). No differences were found in the TRPV1 KO mice.* ***B****, Histogram showing the mean amplitude of bladder voiding contractions of WT and TRPV1 knockout mice treated with saline or NGF. The amplitude of bladder reflex contractions remained unchanged in WT and TRPV1 KO mice despite the treatment.* ***C****, Histogram depicting the mean area under the curve (AUC) of bladder voiding contractions of WT and TRPV1 KO mice treated with saline or NGF. The AUC was increased in WT mice treated with NGF, when compared with saline-treated WT mice (*P < 0.05). No changes were observed in the AUC of TRPV1 KO mice.*



per minute (Figs 2A,C,3A). No differences were found in the AUC between the two groups (Fig. 3C). Treatment with NGF significantly increased the frequency of voiding contractions in WT mice to 1.2 ± 0.4 per minute ($P < 0.05$ vs saline treatment; Figs 2B,3A). In NGF-treated KO mice, the frequency of voiding contractions was 0.5 ± 0.1 per minute (Figs 2D,3A). The amplitude of voiding contractions of WT and TRPV1 KO mice treated with saline were 27.0 ± 5.1 cmH_2O and 23.4 ± 4.2 cmH_2O, respectively (Fig. 3B). Repeated NGF administration both in WT and TRPV1 KO mice did not alter the amplitude of voiding contractions, the values being 23.5 ± 4.8 cmH_2O and 22.8 ± 2.3 cmH_2O, respectively (Fig. 3B). Prolonged NGF treatment also resulted in an increase of the AUC, both in WT mice ($P < 0.05$ vs saline treatment) and KO mice (Fig. 3C).

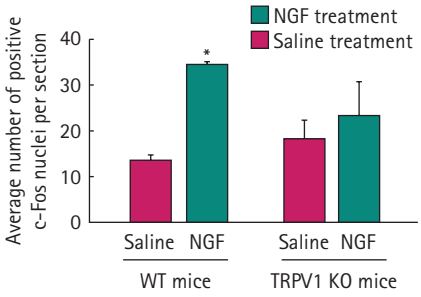
We also found significant expression of the surrogate marker of noxious sensory input c-Fos in neuronal nuclei in L6 spinal sections. The number of immunoreactive cells was very low in control WT and TRPV1 KO mice (13.4 ± 1.4 and 18.4 ± 4.0; Fig. 4). Treatment with NGF significantly increased the number of positive nuclei in WT animals (34.6 ± 0.5; $P < 0.05$ vs saline-treated WT mice; Fig. 3) but not in TRPV1 KO mice (23.3 ± 7.3; Fig. 4).

Expression of TrkA in the L6 segment of the spinal cord and respective DRG was assessed by immunohistochemistry. TrkA staining intensity was similar between WT and TRPV1 KO mice both in the L6 spinal cord segment (Fig. 5A,C,E) and DRG (Fig. 5B,D,F). This confirmed that expression of the high-affinity NGF receptor TrkA was identical in WT and TRPV1 KO mice.

DISCUSSION

Classically, the NGF and TRPV1 systems are seen as key players in nociception and visceral sensitization under inflammatory circumstances but have largely been regarded as parallel rather than linked pathways. However, a role of TRPV1 in bladder overactivity and noxious input associated with increased exposure to exogenous NGF was found here. In fact, TRPV1 KO mice, in contrast to WT mice, did not develop thermal hypersensitivity, significant signs of altered bladder function or spinal cord c-Fos overexpression after prolonged administration of NGF. Of note, this was not the result of altered expression of NGF high-affinity TrkA receptors, which we showed to be similarly expressed in WT and KO mice. Hence, NGF-induced bladder overactivity and increased noxious input depend on the interaction of NGF with

*FIG. 4. Histogram showing the average number of positive c-Fos nuclei in spinal cord sections after saline or nerve growth factor (NGF) treatments. NGF treatment produced a significant increase of c-Fos expression only in wild-type (WT) mice (*P < 0.05) in comparison with saline. No differences were found in the transient receptor potential vanilloid 1 knockout (TRPV1 KO) mice group.*



TRPV1. This indicates that the NGF/TRPV1 interaction, shown after acute NGF administration in somatic tissues [12], also stands after prolonged NGF administration in a visceral model of bladder overactivity and pain. Of note, bladder changes induced by repeated NGF administration were accompanied by the development of thermal hyperalgesia, a novel fact described here.

Previous models of TRPV1 sensitization by G-protein-coupled receptor agonists, such as those for prostaglandins and bradykinin, have implied phosphatidyl-inositol-4,5-bisphosphate (PIP_2) degradation and protein kinase C activation in TRPV1 regulation [12,15]. PIP_2 is a molecule that maintains TRPV1 under tonic inhibition. Interestingly, PIP_2 cleavage can be enhanced by NGF. Activation of TrkA receptors by NGF may lead to phospholipase C. Moreover, binding of NGF to TrkA also leads to activation of the phosphatidylinositol-3-kinase and extracellular signal-regulated kinase 1 and 2 pathways, which further results in facilitation of TRPV1 activity [16,17]. Whereas these are believed to be short-term cellular responses induced by acute administration to NGF, our findings suggest that they remain operative upon prolonged exposure to elevated NGF concentrations.

TRPV1 is unlikely to mediate all NGF responses and, similarly, NGF may not be the only pathway inducing TRPV1 sensitization [16,18,19]. In fact, the lipidic inflammatory mediators *N*-arachidonoyl-ethanolamine, also known as anandamide, *N*-arachidonoyl-

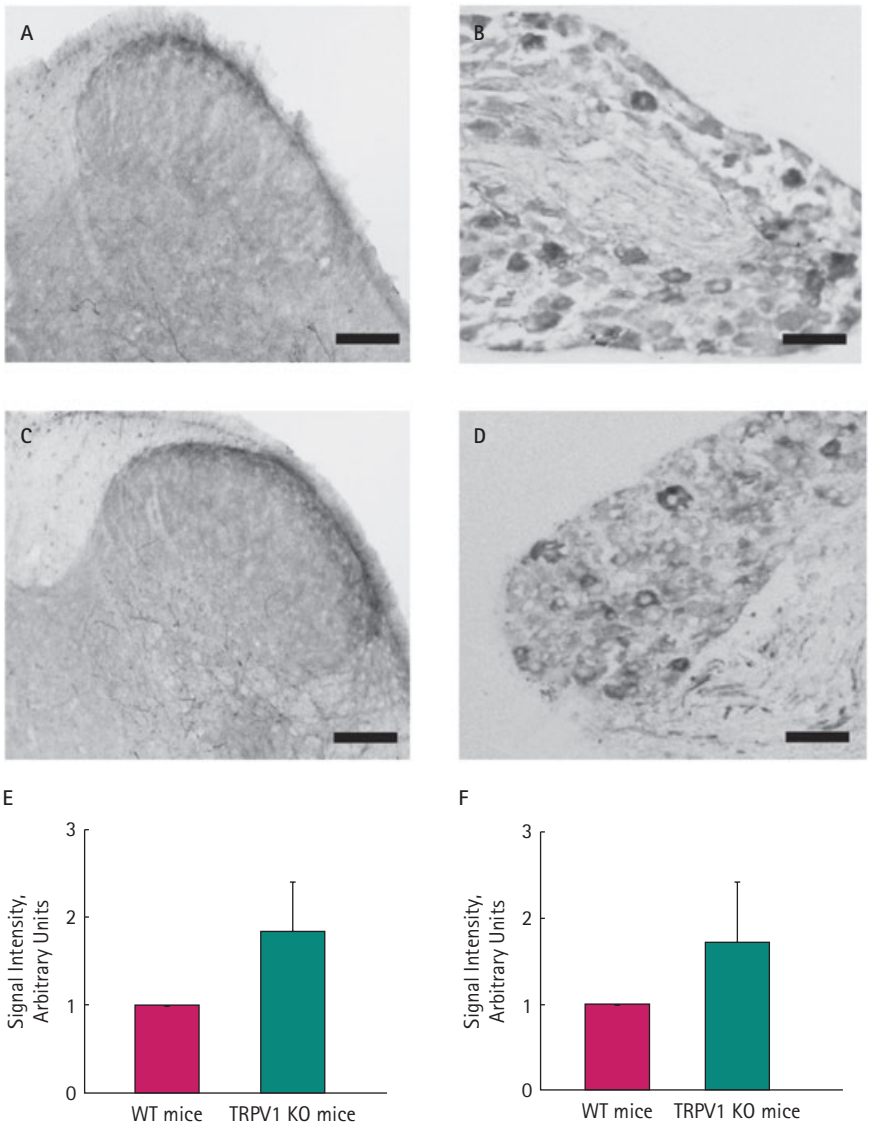
dopamine, *N*-oleoyldopamine, eicosanoid acids and leukotrienes may also participate in TRPV1 sensitization [20]. In addition, it should be recalled that a decrease in the inflamed tissue pH is known to reduce the heat threshold of TRPV1 from around 43 °C down to physiological temperatures [21]. Future studies may be relevant to elucidate a possible cumulative relation between lipid mediators, protons and NGF in the process of TRPV1 activation.

Another mechanism of TRPV1 sensitization comprises down-regulation on the expression of inhibitory TRPV1 splice variants. Using the cyclophosphamide model of bladder inflammation, Charrua *et al.* [22] showed that the expression of the dominant negative splice variant TRPV1b was decreased in sensory afferents innervating the bladder. Although tempting, it cannot be concluded that NGF regulates TRPV1 alternative splicing.

There is still some debate regarding the role of TRPV1 for normal micturition. In the present study, we observed the presence of non-voiding oscillations of the bladder wall that preceded voiding bladder contractions. This is in agreement with observations made by other investigators [23], who also reported the presence of similar non-voiding contractions. However, it contrasts with previous results from our own group [1]. This is probably the result of the use of a less sensitive pressure detector, which was not used in the present study. The true reasons can only be speculated but may be ascribed to compensatory responses of the bladder or the nervous system to the congenital absence of the TRPV1 receptor. It is possible that the natural presence of the receptor may dampen those non-voiding contractions by modulation of bladder sensory afferents, urothelial cells or detrusor muscle fibres [23]. Despite the presence of these non-voiding contractions, bladder function was normal as indicated by similar frequency of voiding bladder contractions and AUC. This indicates that TRPV1 should be seen as a receptor essential for bladder dysfunction mainly related with inflammation and plays a modest role in normal bladder function [1,24].

Our findings may have profound implications in re-directing therapeutic research. There are high expectations of the use of agents interfering with NGF signalling, namely the use of tanezumab, an anti-NGF monoclonal antibody that prevents this neurotrophin from binding to its cognate receptor TrkA. A recent phase 2 study in patients with interstitial cystitis/bladder pain syndrome showed that NGF sequestration improved bladder pain at a high toll of adverse events, which included vertigo, paraesthesia and hyperesthesia [25]. In addition, in other trials with the same drug several subjects developed bone necrosis requiring total joint replacement [26]. This led the Food and Drug Administration to suspend the clinical trials involving tanezumab. In this scenario, TRPV1 antagonists appear as a much more attractive therapy. These drugs effectively improve bladder overactivity and noxious input associated with bladder inflammation [24,27,28]. Nevertheless, they still present some drawbacks, such as the risk of hyperthermia and increasing the extension

FIG. 5. ***A*****–*****D****, Photomicrographs of TrkA receptor expression in L6 segment of the spinal cord and respective dorsal root ganglion (DRG) of wild-type (WT) and transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice. In the spinal cord, TrkA receptor is present in laminae I and II in both WT (****A****) and TRPV1 KO (****C****) mice. In the DRG, TrkA receptor is expressed by small and medium-sized neurons of WT (****B****) and TRPV1 KO mice (****D****). Scale bar 50 μm. (****E****) Graph bar depicting the mean intensity of TrkA receptor in the L6 spinal cord and respective DRG in WT and TRPV1 KO mice. TrkA receptor intensity was similar in WT and TRPV1 KO mice, both in spinal cord (****E****) and DRG (****F****).*



of ischaemic tissue after coronary obliteration.

In conclusion, our results indicate that the interaction between NGF and TRPV1 is crucial for visceral overactivity and pain associated with prolonged exposure to NGF. TRPV1 therefore serves as an important bottleneck for chronic inflammatory pain, as well as visceral pain and lower urinary tract symptoms, a major reason why patients seek medical help. In this context, the development of TRPV1 antagonists assumes a clear therapeutic interest.

ACKNOWLEDGEMENTS

Financial support was given by InComb FP7 HEALTH project no 223234; Barbara Frias is supported by an FCT scholarship SFRH/BD/63225/2009 from Fundação para a Ciência e Tecnologia (Portugal).

CONFLICT OF INTEREST

Martin C. Michel is an employee of Boehringer Ingelheim. Francisco Cruz is a consultant for Astellas.

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Abbreviations: **TRPV1**, transient receptor potential vanilloid 1; **KO**, knockout; **NGF**, nerve growth factor; **WT**, wild-type; **ABC**, avidin–biotin complex; **PBST**, PBS containing Triton X-100; **AUC**, area under the curve; **DAB**, 3,3′-diaminobenzidine tetrahydrochloride as chromogen; **DRG**, dorsal root ganglion; **PIP_2**, phosphatidyl-inositol-4,5-bisphosphate.

Final Considerations

a I·yê ōfI·ŘŘŜN LJI·ŰK2f2ƎƛŜăΣ ădzŐK I·ă ŰKŜ 2ØŜNJI·ŐŰƛØŜ ōfI·ŘŘŜN ăèyŘN2YˇŜ óh ! . ú I·yŘ ySdzN2ƎŜyƛŐ ŘŜŰNdzăzN 2ØŜNJI·ŐŰƛØƛŰê ób5húΣ I·NŜ ŐKI·NJI·ŐŰŜNƛÎŜŘ ōè I· ăŜNƛŜă 2Ŧ [¦ ¢ ăèYˇLJŰ2Yˇă ó[¦ ¢{ú ŰKI·ŰƛyŐfdzŘŜ Ø2ƛŘƛyƎ ₣NŜIjdzŜyŐè I·yŘ dzNƎŜyŐè ó ! ōNJI·Yˇă ŜŰ I·f¢Σ нллнΣ I I·ăKƛY I·yŘ ! ōNJI·YˇăΣ нллтú¢ LyˇLJI·ŰƛŜyŰă ădz₣₮ŜNƛyƎ ₣NZY ōfI·ŘŘŜN LJI·ƛyˇăèyŘN2YˇŜкƛyŰŜNăŰƛŰƛI·f ŐèăŰƛŰƛă ó . t{кL / úΣ LJI·ƛyˇƛă I·fă2 I·ăă2ŐƛI·ŰŜŘ ØƛŰK ōfI·ŘŘŜN ₣ƛfƛyƎ ó I I·yŷ2 ŜŰ I·f¢Σ нлммú¢ ¢KŜ ăŜyã2NŵÈ yI·ŰdzNŰŜ 2Ŧ ŰKŜăŜ [¦ ¢{ YˇI·è NŜLJNŰŜăŜyŰ ₣dzyŐŰƛ2yI·f I·fŰŜNJI·Űƛ2yã 2Ŧ ōfI·ŘŘŜN I·₣₮ŜNŰŜyŰă YˇŜŘƛI·ŰŜŘ ōè YˇI·yê ₣I·ŐŰ2NƛΣ ƛyŐfdzŘƛyƎ b¢ă¢ ¢KŜăŜ ŰN2LJKƛŐ ₣I·ŐŰ2NăΣ LJI·NŰƛŐdzfI·N₮ê bDC I·yŘ . 5bCΣ I·NŜ YˇI·ăŰŜNJ NŜƎdzfI·Ű2Nă 2Ŧ ySdzN2yI·f LJfI·ăŰƛŐƛŰèΣ ō2ŰK ƛyˇŰKŜ LJŜNƛLJKŜNJI·f I·yŘ ŐSyŰNJI·f ySN Ø2dză ăèăŰŜY ótŜÎŜŰ I·yŘ aŏa I·Ŕ2yΣ нллсú¢ ! fŰK2dzƎK ŰKŜ ƛYˇLJ2NŰI·yŐŜ 2Ŧ bDC ƛyˇ [¦ ¢ ŘèăŦdzyŐŰƛ2yˇƛă I·fNŜI·Řè NŜŐ2ƎyƛÎŜŘ óhŐK2ŘyƛŐ]è ŜŰ I·f¢Σ нлммúΣ ŰKŜ ₣ƛySˇYŜŐKI·yƛăYˇă 2Ŧ bDC-ŘŜLJSyŘŜyŰ ōfI·ŘŘŜN KèLJŜNJI·ŐŰƛØƛŰê ØŜNŜ y2ŰΣ ŐfŜI·N¢ LyˇI·ŘŘƛŰƛ2yΣ ŰKŜ LJ2ŰŜyŰƛI·f N2fŜ 2Ŧ . 5bC ƛyˇōfI·ŘŘŜN ŘèăŦdzyŐŰƛ2yˇØI·ă dzy]y2øyˇI·Ű ŰKŜ ŰƛYˇŜ ŰKŜăŜ ăŰdzŘƛŜă ØŜNŜ ƛyƛŰƛI·ŰŜŘ¢

1. BDNF role in bladder hyperactivity and referred pain during cystitis

LŰ ƛă ØŜff]y2øyˇ ŰKI·Ű ŐKN2yƛŐ ŐèăŰƛŰƛăΣ ƛyŘdzŐŜŘ ōè ƛyŰNJI·LJŜNƛŰ2yŜI·f / ¸t ƛy?ŜŐŰƛ2yΣ ƛă ŐKI·NJI·ŐŰŜNƛÎŜŘ ōè ōfI·ŘŘŜN KèLJŜNJI·ŐŰƛØƛŰê I·yŘ ƛyŐNŜI·ăŜŘ LJI·ƛyˇó[I·yŰŜNƛ- aƛySŰ ŜŰ I·f¢Σ мффpΣ 5ƛyƛă ŜŰ I·f¢Σ нллпΣ / NdzÎ ŜŰ I·f¢Σ нллpI·ú¢ / èăŰƛŰƛă ƛă I·ŐŐ2YˇLJI·yƛŜŘ ōè I·yˇdzLJNŜƎdzfI·Űƛ2yˇƛyˇbDC fŜØŜfă ó[2øŜ ŜŰ I·f¢Σ мффтΣ .?2NfƛyƎ ŜŰ I·f¢Σ нллмΣ DdzŜNƛ2ă ŜŰ I·f¢Σ нллсΣ DdzŜNƛ2ă ŜŰ I·f¢Σ нллуú I·yŘ ōf2Ő]I·ŘŜ 2Ŧ bDC I·ŐŰƛ2yˇ ŜƛŰKŜN ōè ăŐI·ØŜyƎƛyƎ I·ƎŜyŰă 2N ¢N] I·yŰI·Ǝ2yŋăŰă I·YˇŜf‰2NJI·ŰŜ ōfI·ŘŘŜN KèLJŜNJI·ŐŰƛØƛŰê I·yŘ NJŜ₮ŜNNJŜŘ LJI·ƛyˇó I dz ŜŰ I·f¢Σ нллрΣ DdzŜNƛ2ă ŜŰ I·f¢Σ нллуú¢ ¢KŜ ƛyØ2fØŜYˇŜyŰ 2Ŧ . 5bC ƛyˇØƛăŐŜNJI·f LJI·ƛyˇ KI·Ř I·fNŜI·Řè ōŜŜyˇădzƎƎŜăŰŜŘ ƛyˇŰKŜ LJI·yŐNŜI·ă ó½Kdz ŜŰ I·f¢Σ нллмΣ I dzƎKŜă ŜŰ I·f¢Σ нлммú ōdzŰΣ I·Ű ŰKŜ ōŜƎƛyŷƛyƎ 2Ŧ ŰKŜăŜ ăŰdzŘƛŜăΣ ŰKŜ Ő2yŰNƛōdzŰƛ2yˇ2Ŧ . 5bC Ű2 ōfI·ŘŘŜN KèLJŜNJI·ŐŰƛØƛŰê I·yŘ LJI·ƛyˇŘdzNƛyƎ Őèă ŰƛŰƛă ø I·ă dzy]y2øy¢ ¢Kƛă ø I·ă I·ŘŘNŜăŜŘ ƛyˇLJdzōfƛŐI·Űƛ2yã LL I·yŘ LLL¢

wŜădztŰă ăK2ø ŰKI·Ű ƛyŰNJI·ŰKŜŐI·f I·ŘYˇƛyƛăŰNJI·Űƛ2yˇ 2Ŧ . 5bC ƛyŘdzŐŜŘ Ř2ăŝ-ŘŜLJŜyŘŜyŰ ōfI·ŘŘŜN KèLJŜNJI·ŐŰƛØƛŰê I·yŘ NJŜ₮ŜNNJŜŘ LJI·ƛyˇóLJdzōfƛŐI·Űƛ2yˇ LLLú¢ ¢KŜăŜ Ŝ₮₮ŜŐŰă I·LJLJŜI·NJŜŘ IjdzƛŐ]fè I·₮ŰŜNJ . 5bC ƛy?ŜŐŰƛ2yˇ I·yŘ ØŜNJŜ ăK2NŰ-fI·ăŰƛyƎ¢ ¢KŜ NJŜI·ă2yˇ ₮2N ŰKƛă YˇI·è ōŜ I·ăÕNƛōŜŘ Ű2 ŰKŜ Ř2øyăŰNJŜI·Yˇ ŜØŜyŰă 2ŐŐdzNNJƛyƎ ƛyˇăŜŐ2yŘ 2NJŘŜNJ yŜdzN2yã¢ . 5bC ōƛyŘƛyƎ Ű2 ălJƛyI·f ¢N] . NJŜŐŜLJŰ2N ƛyˇŰKŜ ălJƛyI·f Ő2NJŘ tŜI·Řă Ű2 ŰKŜ IjdzƛŐ] I·ŐŰƛØI·Űƛ2yˇ 2Ŧ ŰKŜ 9wY м I·yŘ н ăƛƎyI·fƛyƎ LJI·ŰKøI·è ó[ŜØŜNJ ŜŰ I·f¢Σ нллоōΣ {fI·Ő] ŜŰ I·f¢Σ нллпΣ {fI·Ő] ŜŰ I·f¢Σ нллрúΣ I·ă ƛŰ KI·Ř I·fNŜI·Řè ōŜŜyˇ2ōăŜNJØŜŘ dzăƛyƎ ŰKŜ ălˇYˇŜ YˇƎŘŜf 2Ŧ Ðèă ŰƛŰƛă ó / NdzÎ ŜŰ I·f¢Σ нллpI·ú¢ [ƛ]Ŝ . 5bC-ƛyŘdzŐŜŘ ōfI·ŘŘŜN KèLJŜNJI·ŐŰƛØƛŰê I·yŘ LJI·ƛyΣ ălJƛyI·f 9wY I·ŐŰƛØI·Űƛ2yˇƛyŘdzŐŜŘ ōè ōfI·ŘŘŜN ăŰƛYˇdzfI·Űƛ2yˇăă I·fă2 I· ōNƛŜŦ ŜØŜyŰ ó / NdzÎ ŜŰ I·f¢Σ нллpI·úΣ LJ2ăăƛōfè ŘdzŜ Ű2 ŰKŜ I·ŐŰƛØI·Űƛ2yˇ2Ŧ ălJŜŐƛ₮ƛŐ LJK2ălJKI·ŰI·ăŜă ŰKI·Ű YˇƎŘdzfI·ŰŜ ŰKŜ 9wY LJI·ŰKøI·è ó adzŘI· ŜŰ I·f¢Σ мффcΣ / I·dzyŰ I·yŘ YŜèăŜΣ нлмоú 2N ŰKŜ IjdzƛŐ] ŘŜƎNJI·ŘI·Űƛ2yˇ 2Ŧ . 5bC ƛyˇ ŰKŜ ăèyI·LJŰƛŐ ŐfŜ₮Ű ōè YˇI·ŰNƛÈ LJNƎ2ŰŜI·ăŜă ó tŜÎŜŰ ŜŰ I·f¢Σ нллнŐú¢

Chronic administration of BDNF to intact animals resulted in allodynia in the lower abdomen and hindpaw (publication III), reflecting the occurrence of central sensitization, a mechanism associated with somatic and visceral pain (Miranda et al., 2007, Latremoliere and Woolf, 2009), known to depend on BDNF (Obata and Noguchi, 2006, Ren and Dubner, 2007, Li et al., 2008, Merighi et al., 2008). The role of BDNF in chronic visceral pain may be related with ERK-dependent changes in spinal gene expression. Upon activation by BDNF, ERK can translocate to the cell nucleus and induce the expression of pronociceptive genes such as the tachykinin receptor NK1 and prodynorphin (Ji et al., 2002b). In addition, ERK may also phosphorylate specific NMDA subunits (Slack and Thompson, 2002, Slack et al., 2004, Slack et al., 2005) and the $Kv_{4.2}$ potassium channel (Hu et al., 2006, Hu and Gereau, 2011), further facilitating pain processing and bladder hyperactivity (Cruz et al., 2005a) at the spinal cord level.

In what concerns bladder function, chronic BDNF treatment in intact animals did not change bladder reflex activity (publication III). This was an unexpected finding since chronic treatment with BDNF lead to allodynia. A possible explanation may reside in the fact that exogenous BDNF can facilitate the release of GABA through activation of TrkB receptor in the dorsal horn (Pezet et al., 2002a, Lever et al., 2003a). The enhancement of the spinal GABAergic system may lead to the inhibition of bladder sensory input transmission, thereby preventing the development of bladder hyperactivity. In addition, it should be remembered that rats receiving chronic BDNF did not present bladder inflammation. This may indicate that bladder hyperactivity requires a peripheral inflammatory insult.

Given the effects of BDNF on bladder function and cutaneous sensitivity of intact animals, the role of BDNF in rats with CYP-induced cystitis was investigated. BDNF was upregulated both in the spinal cord and urinary bladder of inflamed rats. This was accompanied by bladder hyperactivity and obvious behavioral signs of pain (publications II and III), together with prominent expression of c-Fos and phosphorylated ERK, established spinal neuronal markers of noxious input (Coggeshall, 2005, Ji et al., 2009). Peripheral and central BDNF sequestration effectively improved bladder function and pain levels, further implying this NT in visceral pain and dysfunction. As following BDNF administration, the activation of the ERK pathway is also a key feature here as BDNF sequestration was accompanied by a down regulation in the number of spinal phosphoERK positive cells (publications II and III).

In both studies, the same BDNF scavenger, the recombinant protein TrkB-$Ig_{2,}$ was used. The amount necessary to reduce pain and bladder dysfunction was much smaller when given via intrathecal injection (publication III). In addition, neither intrathecal nor intravenous BDNF sequestration reduced inflammation suggesting that BDNF does not play a role in the

ƛyŧfIꞏϒϒIꞏŭ2₦èŏ2ϒLJ2ySyύ 2ŦŏëăύƛŭƛăΦ ¢Kƛã ăΰ₦2y∃fè ădz∃∃Ŝăŭă ŭKIꞏŭ . 5bC Ŏ2yΰ₦ƛōdzύŜă ŭ2 ØƛăŎŜ₦IꞏfLJIꞏƛyˇ IꞏyŘ ōfIꞏŘŘŜ₦ KèLJŜ₦IꞏŎŭƛØƛúè Iꞏŭ ŭKŜ ŎŜyΰ₦IꞏfySdzØ2dzã ăèăŭŜYˇ ótŜÎŜύ Ŝύ IꞏfΦΣ нллнŎΣ tŜÎŜύ IꞏyŘ aŎaIꞏK2yΣ нллcύΣ ƛyˇŎ2yΰ₦Iꞏăΰ ØƛΰK ŭKŜ Ŝăΰ Iꞏōfăăk ŜŘ LJŜ₦ƛLJKŜ₦IꞏfW2fŜ 2Ŧ bDC óhŎK2ŘyƛŎ]è Ŝύ IꞏfΦΣ нлммΣ hŎK2ŘyƛŎ]è Ŝύ IꞏfΦΣ нлмнΣ /₦dzÎΣ нлмоύΦ

2. BDNF role in the emergence and maintenance of Neurogenic Detrusor Overactivity (NDO)

{LJƛyIꞏf Ŏ2₦Ř ƛyʔdz₦è ƛyŘdzŎŜă LJ₦2Ŧ2dzyŘ ŎKIꞏy∃Ŝă ƛyˇōfIꞏŘŘŜ₦ ŦdzyŎŭƛ2yˇ IꞏyŘ ∃ŜyŜ₦IꞏúŜă b5hΣ ŭKIꞏŭ ŭèLJƛŎIꞏffè fŜIꞏŘă ŭ2 dz₦ƛyIꞏ₦è ƛyŎ2yŭƛySyŎŜ ó/₦dzÎ IꞏyŘ /₦dzÎΣ нлммύΦ ¢KŜ Ŝăŭ Iꞏōfăk KYˇŜyΰ 2Ŧ b5h ƛă YˇIꞏƛyfè Iꞏŭύ₦ƛōdzύIꞏōfŜ ŭ2 ŭKŜ ₦Ŝ2₦∃IꞏyƛÎIꞏŭƛ2yˇ 2Ŧ ŭKŜ ySdz₦Iꞏf LJIꞏŭKøIꞏèă ₦Ŝ∃dzfIꞏŭƛy∃ ōfIꞏŘŘŜ₦ IꞏyŘ dz₦ŜúK₦Iꞏf ăLJKƛyŎúŜ₦ ŦdzyŎŭƛ2yˇfŜIꞏŘƛy∃ ŭ2 ŭKŜ ₦ŜIꞏLJLJŜIꞏ₦IꞏyŎŜ 2Ŧ LJŜ₦ƛySIꞏf-ŭ2-ōfIꞏŘŘŜ₦ IꞏyŘ ōfIꞏŘŘŜ₦çύ2-ōfIꞏŘŘŜ₦ ₦ŜŦfŜÈŜă óŘŜ D₦2Iꞏŭ IꞏyŘ ¸2ăKƛYˇdz₦IꞏΣ нллcύΦ Lύ ăă IꞏăădzYˇŜŘ ŭKIꞏŭ b¢ă Iꞏ₦Ŝ ƛyØ2fØŜŘ ƛyˇ ŭKƛă ₦ŜIꞏ₦₦IꞏyƎŜYˇŜyΰ 2Ŧ fdzYˇō2ăIꞏŎ₦Iꞏf ăLJƛyIꞏf ŎΛ₦ŎdzƛúăΦ ó±ƛÎÎIꞏ₦ŘΣ нллcύΦ ² KŜ₦ŜIꞏă ƛΰ ăă]yZøyˇ ŭKIꞏŭ ƛYˇdzy2yŜdzύ₦IꞏfƛÎIꞏŭƛ2yˇ 2Ŧ bDC ₦ŜŘdzŎŜă b5h IꞏyŘ ŘŜŭ₦dză2₦-ăLJKƛyŎúŜ₦-Řèăăèy$₦∃ƛIꞏ ó5{5ú ó{Ŝ]ƛ Ŝύ IꞏfΦΣ нллнΣ {Ŝ]ƛ Ŝύ IꞏfΦΣ нллпύΣ y2 ŘIꞏúIꞏ øIꞏă IꞏØIꞏƛfIꞏōfŜ Ŏ2yŎŜ₦yƛyƎ ŭKŜ ₦2fŜ 2Ŧ . 5bC ƛyˇ b5hΣ ŘŜăLJƛúŜ ŭKŜ ŘŜYˇ2yãú₦Iꞏŭƛ2yˇ ŭKIꞏŭ . 5bC ăă ƛyØ2tØŜŘ ƛyˇ ₦ŜƎŜySy$₦Iꞏŭƛ2yˇ 2Ŧ ySdz₦2yIꞏf ú₦IꞏŎŭă Ŧ2ff2øƛyƎ ăLJƛyIꞏf Ŏ2₦Ř ú₦IꞏdzYˇIꞏ óbIꞏYˇƛ]ƛ Ŝύ IꞏfΦΣ нлллΣ ±IꞏØ₦Ŝ] Ŝύ IꞏfΦΣ нллтύΦ ¢Kdzăΰ Ŧ2ff2øƛyƎ 2dz₦ ŘŜYˇ2yãú₦Iꞏŭƛ2yˇ ŭKIꞏŭ . 5bC LJIꞏ₦ŭƛŎƛLJIꞏŭŜă ƛyˇ ƛyŧfIꞏYˇYˇIꞏŭƛ2y-ƛyŘdzŎŜŘ ōfIꞏŘŘŜ₦ KèLJŜ₦IꞏŎŭƛØƛúèΣ øŜ ƛyØŜăŭƛ∃IꞏúŜŘ ƛŦ ŭKƛă bø øIꞏă IꞏfăƛϒLJ2₦ύIꞏyΰ Ŧ2₦ b5hΦ

{/L ăΰ₦2y∃fè ƛYˇLJIꞏƛ₦ŜŘ ōfIꞏŘŘŜ₦ ŦdzyŎŭƛ2yˇIꞏŭ 2yŜ øŜŜ] IꞏŦûŜ₦ {/LΣ øƛŭK IꞏyƛYˇIꞏfă ăK2øƛyƎ ăƛ∃yã 2Ŧ ăLJƛyIꞏf ăK2Ŏ]Σ ŎKIꞏ₦IꞏŎûŜ₦ƛÎŜŘ ōè ōfIꞏŘŘŜ₦ Iꞏ₦ŜŦfŜÈƛIꞏ IꞏyŘ dz₦ƛyIꞏ₦è ₦ŜûŜyŭƛ2yˇ óLJdzōfƛŎIꞏŭƛ2yˇ L±úΦ !ff IꞏyƛYˇIꞏfă KIꞏŘ ŭ2 ōŜ ădzōYˇƛúûŜŘ ŭ2 ŘIꞏƛfè IꞏōŘ2YˇƛyIꞏf Ŏ2YˇLJ₦Ŝăăƛyˇ ŭ2 ₦ŜYˇ2ØŜ dz₦ƛySΦ C2dz₦ øŜŜ]ă fIꞏúŜ₦Σ IꞏffIꞏyƛYˇIꞏfă LJ₦ŜăSyύŜŘ b5hΣ øƛúK Iꞏ YˇIꞏ₦]ŜŘ ƛyŎ₦ŜIꞏăŜ ƛyˇ ŭKŜ Ŧ₦ŜIjdzŜyŎè IꞏyŘ IꞏYˇLJfƛúdzŘŜ 2Ŧ ōfIꞏŘŘŜ₦ Ŏ2yΰ₦IꞏŎŭƛ2yã Iꞏă øŜff Iꞏă 2Ŧ ŭKŜ ƛyΰ₦IꞏØŜăƛŎIꞏf LJ₦ŜăădzŵŜ óLJdzōfƛŎIꞏŭƛ2yˇ L±úΦ ¢Kƛă øIꞏă IꞏŎŎ2YˇLJIꞏyƛŜŘ ōè Iꞏ ŭƛYˇŜ-ŘŜLJŜyŘŜyΰ ăLJ₦2dzŭƛyƎ 2Ŧ ăŜyã2₦è IꞏŦŦŜ₦Syΰă IꞏyŘ ƛyŎ₦ŜIꞏăŜ ƛyˇăLJƛyIꞏf .5bCΦ t2ŭŜyΰƛIꞏf ă2dz₦ŎŜă 2Ŧ ăLJƛyIꞏf .5bC ƛyŎfdzŘŜ ăLJƛyIꞏf ƛyΰŜ₦yŜdz₦2yã ó aƛŎKIꞏŜf Ŝύ IꞏfΦΣ мффтύΣ Iꞏăΰ₦2ŎèúŜă IꞏyŘ YˇƛŎ₦2∃fƛIꞏ ó/2dzff Ŝύ IꞏfΦΣ нллрΣ tƛySIꞏdz IꞏyŘ [IꞏŎ₦2ƛÈΣ нллтύΦ .fIꞏŘŘŜ₦ ăŜyã2₦è IꞏŦŦŜ₦SyΰăIꞏ₦Ŝ IꞏfăƛLJ2ŭŜyΰƛIꞏf ă2dz₦ŎŜă 2Ŧ .5bC Iꞏă ŭKƛă bø ƛă LJ₦2ŘdzŎŜŘ ƛyˇ ŭKŜ ōfIꞏŘŘŜ₦ ó[ƵYˇIꞏúÎăŎK Ŝύ IꞏfΦΣ мфффΣ [ƵYˇIꞏúÎăŎK Ŝύ IꞏfΦΣ нллрΣ tƛyΰ2 Ŝύ IꞏfΦΣ нлмлIꞏύ IꞏyŘ dzLJ₦Ŝ∃dzfIꞏúŜŘ Ŧ2ff2øƛyƎ {/L ó±ƛÎÎIꞏ₦ŘΣ нллύΦ Lΰƛ ƛă LJfIꞏdzăƛōfŜ ŭKIꞏŭ .5bC ƛă dzLJΰIꞏ]Syˇ ƛyˇ ŭKŜ ōfIꞏŘŘŜ₦ IꞏyŘ ₦ŜΰΣ₦2∃₦IꞏŘŜfè ΰ₦IꞏyãLJ2₦ύŜŘ ŭ2 ōŜ ₦ŜfŜIꞏăŜŘ Iꞏŭ ŭKŜ ăLJƛyIꞏf Ŏ2₦Ř ó½K2dz IꞏyŘ wdzăKΣ мффсΣ aƛŎKIꞏŜf Ŝύ IꞏfΦΣ мффтύΦ

The increase in BDNF levels and the emergence of NDO suggested a causal relation between the two. To clarify this, SCI rats were submitted to BDNF sequestration by TrkB-Ig_2. Treatment was initiated immediately after lesion. Surprisingly, BDNF scavenging resulted in earlier NDO emergence and axonal sprouting of CGRP-positive sensory afferents at the L5-L6 spinal cord segment. This suggests that BDNF may have a protective role by delaying the emergence of NDO during the course of disease. It is possible that, following SCI, the upregulation of spinal BDNF may serve to modulate axonal sprouting that occurs as a response to the high levels of NGF. Following SCI and during the establishing of NDO, NGF levels are upregulated (Seki et al., 2002) and produce a growth-promoting effect on CGRP-positive sensory afferents (Krenz and Weaver, 1998, Weaver et al., 2001, Cameron et al., 2006). BDNF seems to exert an inhibitory effect on NGF-induced growth of sensory neurons, as already demonstrated by other investigators (Kimpinski et al., 1997, Gavazzi et al., 1999, Soril et al., 2008).

To confirm the protective effects of BDNF on bladder function in SCI rats, BDNF was administered to these animals for four weeks. Treatment was initiated immediately after lesion. Improvement of bladder reflex activity, restricted to a reduction in the intravesical pressure, was only found four weeks after the beginning of treatment with the lowest dose (publication IV). This suggests that BDNF is not the only factor involved in NDO establishing. The protective effects of BDNF may be related with changes in gamma-aminobutyric acid (GABA)-dependent neurotransmission at the spinal cord level. This inhibitory neurotransmitter is an important depressor of bladder function (Igawa et al., 1993, Miyazato et al., 2003). In fact, the expression of glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis, is reduced in the spinal cord and lumbosacral dorsal root ganglia in SCI-animals with bladder dysfunction (Miyazato et al., 2008a, b). Both NDO and DSD were reduced following intrathecal injection of $GABA_A$ or $GABA_B$ receptor agonists and transgenic upregulation of GAD activity (Miyazato et al., 2008a, b, Miyazato et al., 2009), further stressing the importance of GABA in modulation of bladder function. As the release of GABA at the spinal cord may be induced and potentiated by BDNF (Pezet et al., 2002a, Bardoni et al., 2007, Carrasco et al., 2007), it is likely that chronic administration of this NT may have modulated the spinal GABAergic system.

Results obtained show that BDNF sequestration in animals with established NDO resulted in improvement of bladder function, with a clear decrease in the frequency and amplitude of bladder reflex contractions. Because spinal ERK activation is very high in rats with established NDO and its inhibition immediately depresses bladder reflex activity (Cruz et al., 2006), similarly to what was observed in the present study, it is very likely that the acute beneficial

ŜŦŦŜŎΩĺă 2Ŧ . 5bC ăŜljdzŜăΩÚN·Ωĺ2y Ò2dzfŘ ōŜ l·ăÕN₳ōŜŘ Ω2 ΩĺKŜ ăøƛŦΩ ƛy1·ŎΩĺØl·Ωĺ2y 2Ŧ ΩĺKl·Ω ăƛƎyl·f1yƎ LJl·ΩĺKøl·è¢

hØŜNl·ff ăƛYƛfl·N NŜădzfΩĺă Kl·ØŜ ōŜŜy ŘŜăÕN₳ōŜŘ ƛy l· Y2ŘŜf 2Ŧ Ř2NälŀfN 2Ω2Ω ƛy?dzNè ØƛΩK . 5bC ăŜljdzŜăΩÚN·Ωĺ2y LJNN2ŘdzÕƛyƎ 2LJLJ2ăƛΩŜ ŜŦŦŜŎΩĺă ŘŜLJSyŘƛyƎ 2y ØKŜΩĺKŜN ƛΩ ø l·ä ƛyƛΩĺ·ΩŜŘ ƛYYŜŘƛl·ΩŜfè l·₮ΩŜN fŜăƛ2y 2N l·₮ΩŜN ΩĺKŜ ăSyã2Nè Řèă₮dzyÕΩĺ2y ø l·ä l·flNŜl·Řè Ŝăΰl·ōflăKŜŘ ówl·YŜN Ŝΰ l·f¢Σ нллтΣ wl·YŜNΣ нлмнú¢ ¢KŜăŜ NŜădzfΩĺă 2ōΏl·ƛySŘ ƛy ΩĺKŜ LJNŜăSyΏ ăΩdzŘè ₮dzNΩĺKŜN Ò2y₮ƛNYl· Řdzl·f N2fŜ ₮2N . 5bC Řdz₦ƛyƎ ΩĺKŜ ŘƛăŜl·ăŜ LJN2ƎNŜăăƛ2y¢

3. Transient receptor potential vanilloid (TRPV1) - a downstream target of NGF

¢KŜ fl·ăΏ ăΩdzŘè ƛyÕfdzŘŜŘ ƛy ΩĺKƛă ΩĺKŜăƛă l·ŘŘNŜăăŜŘ ΩĺKŜ Ř2øyãΩÚNŜl·Y Ωl·NƎŜΩĺă 2Ŧ b¢ăΣ ₮2ÕdzăƛyƎ 2y bDC¢ ! Yl·ƛy Ò2yÕfdzăƛ2y ƛă ΩĺKŜ ŜăΏl·ōflăKYŜyΏ 2Ŧ ΩĺKŜ ŜăăŜyΏƛl·f f1y[ōŜΏøŜŜy ΩĺKŜ bDC l·yŘ ¢wt±M ăèăΏŜYă ƛy ΩĺKŜ Ò2yΏŜÈΏ 2Ŧ ØƛăÕŜNl·f LJl·ƛy l·yŘ ŘèăŦdzyÕΩĺ2y¢ Ly ΩĺKƛă ăΩdzŘèΣ ² ¢ YƛÕŜ ădzōYƛΏΩŜŘ Ω2 ÕKN2yƛÕ ăèăΏŜYƛÕ l·ŘYƛyƛăΩÚNl·Ωĺ2y 2Ŧ bDC LJNŜăŜyΏŜŘ l·y ƛyÕNŜl·ăŜ ƛy ΩĺKŜ ₮NŜljdzŜyÕè l·yŘ l·YLJflΏdzŘŜ 2Ŧ Ø2ƛŘƛy ōfl·ŘŘŜN Õ2yΏNl·ÕΩĺ2yãΣ l·ÕÕ2YLJl·yƛŜŘ ōè l·y ƛyÕNŜl·ăŜ 2Ŧ Õ-C2ă ŜÈLJNŜăăƛ2y l·yŘ ΩĺKŜ ŘŜØŜf2LJYŜyΏ 2Ŧ ΩĺKŜNYl·f KèLJŜNl·fƎŜăƛl· ƛy ΩĺKŜ KƛyŘLJl·øă¢ Ly Ò2yΏNl·ăΏΣ ¢wt±M Yh YƛÕŜ ŘƛŘ y2Ώ NŜăLJ2yŘ Ώ2 bDC ΩNŜl·ΏYŜyΏ l·yŘ Ödzΰl·yŜ2dză ăŜyãƛΏØΏΩèƛyŘ ōfl·ŘŘŜN ₮dzyÕΩĺ2y NŜYl·ƛySŘ dzyÕKl·yƎŜŘ¢ ¢KŜăŜ NŜădzfΩĺă ŘŜY2yãΩÚNl·ΩŜŘ ΩĺKl·Ω ΩĺKŜ ƛyΏŜNLJfl·è ŌŜΏøŜŜy bDC l·yŘ ¢wt±M ƛă ŜăăŜyΏƛl·f ₮2N bDC-ƛyŘdzÕŜŘ ōfl·ŘŘŜN KèLJŜNl·ÕΏØΏèƛyŘ y2ÈƛdzăƛyLJdzΰ óLJdzōfƛÕl·Ωĺ2y ±ú¢ CdzNΏKŜNY2NŜΣ ΏKŜ bDC к¢wt±M ƛyΏŜNl·ÕΏƛ2yΣ ŘŜY2yãΩÚNl·ΩŜŘ ΩĺKl·Ω ₮2ff2øƛyƎ l·ÕdzΰŜ bDC l·ŘYƛyƛăΩÚNl·Ωĺ2y ƛy ă2Yl·Ωĺ2Õ Ωĺăădzŝă ó/Kdzl·yƎ Ŝΰ l·f¢Σ нллмύΣ l·fă2 ăΏl·yŘă l·₮ΩŜN ÕKN2yƛÕ bDC l·ŘYƛyƛăΩÚNl·Ωĺ2y ƛy l· Y2ŘŜf 2Ŧ ōfl·ŘŘŜN KèLJŜNl·ÕΏØΏèƛyŘ ØƛăÕŜNl·f LJl·ƛy óLJdzōfƛÕl·Ωĺ2y ±ú¢

In vitro l·ăăl·èă Kl·ØŜ ăK2øy ΩĺKl·Ω bDC ƛă l·ōfŜ Ω2 NŜƎdzfl·ΩŜ ¢wt±M ŜÈLJNŜăăƛ2y l·yŘ l·ÕΏØΏèè ōè ōƛyŘƛyƎ Ω2 ƛΩă KƛƎK-l·₮₮ƛyƛΩèè NŜŎŜLJΏ2NΣ øKƛÕK ƛyŘdzÕŜă Ř2øyãΩÚNŜl·Y l·ÕΏØΏl·Ωĺ2y 2Ŧ ăƛƎyl·f1yƎ LJl·ΩĺKøl·èăΣ ƛyÕfdzŘƛyƎ a ! tYΣ tY/Σ tLoY 2N t[/ ó.2yŷƛyƎΰ2y l·yŘ aŏbl·dzƎKΏ2yΣ нллоΣ {ΩŜƛy Ŝΰ l·f¢Σ нллсΣ ·dzŜ Ŝΰ l·f¢Σ нллтΣ ½Kdz l·yŘ hÈ₮2NŘΣ нллтú¢ *In vivo*Σ /Kdzl·yƎ l·yŘ Ò2ø2N]ŜNă Kl·Ř l·fă2 ŘŜY2yãΩÚNl·ΩŜŘ ΩĺKl·Ω bDC ƛyΏŜNl·Ŏΰă øƛΩK ¢wt±M Ω2 NŜfŜl·ăŜ ƛΩ ₮N2Y ƛΩă ƛyKƛōƛΩ2Nè Y2ŘdzfΏΩ2N LJK2ăLJKl·ΏƛŘèf-ƛy2ăƛΏ2f-nΣp-ōƛăLJK2ăLJKl·ΩŜ ótLtHú øƛΩK Ò2yãŜljdzŜyÕŜă 2y ΩĺKŜNYl·f ăSyãl·Ωĺ2y ó/Kdzl·yƎ Ŝΰ l·f¢Σ нллмύ¢ ¦yΰƛf ΩĺKŜ LJNŜăŜyΏ ăΩdzŘèΣ ō2ΩK ăèăΏŜYă øŜNŜ ƎŜySNl·ffè NŜƎl·NŘŜŘ l·ă LJl·Nl·ffŜf Nl·ΩĺKŜN ΩĺKl·y flyf[ŜŘ LJl·ΏKøl·ëă ƛyΏŜKŜ Ò2yΏŜÈΏ 2Ŧ ØƛăÕŜNl·f ŘèăŦdzyÕΩĺ2y l·yŘ LJl·ƛy¢

¢wt±M ăă l· LJ2fèY2Řl·f ƛ2y ŎKl·yySfΣ ăŜyãƛΏØŜ Ω2 Ŏl·LJăl·ƛÕƛyΣ y2Èƛdzăƛ KŜl·Ώ l·yŘ f2ø LJl ó¢2YƛyΠ·Ƒl· l·yŘ /l·ΏŜNƛyΠ·Σ нллпύΣ ΏKŜ ΏKNŜăK2fŘ 2Ŧ øKƛÕK ŌŜÕ2YŜă f2øŜN ƛy ƛyfl·YYl·Ώ2Nè Ò2yŘƛΏƛ2yã ó/l·ΏŜNƛyΠ· Ŝΰ l·f¢Σ мффтΣ ¢2YƛyΠ·Ƒl· Ŝΰ l·f¢Σ мффуú¢ Ly ΩĺKŜ ōfl·ŘŘŜNΣ ¢wt±M ăă ŜăăŜyΏƛl·f ₮2N

ƛy₮fl·Y˙Y ׀·ú2₦è õfl·ŘŘŜ₦ KèLJŜ₦׀·ÔúƛØƛúè ׀·yŘ õfl·ŘŘŜ₦-ƎSySŜ₦׀·úŜŘ y2ÈƛdzăƛyŁJdzű ó/K׀·₦₦dz׀· Ŝű ׀·f¢Σ нллтú ōdzű Ř2Ŝă y2ű ăŜŜY˙ ú2 LJ׀·₦úƛÕƛLJ׀·úŜ ƛy˙úKŜ y2₦Y׀·f Y˙ƛÕúdz₦ƛúƛ2y˙ó.ƛ₦ŘŜ₦ Ŝű ׀·f¢Σ ннлнΣ /K׀·₦₦dz׀· Ŝű ׀·f¢Σ нллтú℗ t׀·úƛSyúă ØƛúK .t{κL/ ׀·yŘ b5h LJ₦ŜăŜyúŜŘ ׀·y˙ƛyÕ₦Ŝ׀·ăŜ ƛy˙¢wt±м ƛY˙Ydzy2₦Ŝ׀·ÕúƛØƛúè ƛy˙ ădzōdz₦2úKŜfƛ׀·f ySØŜ ₮ƛōŜ₦ă ó/K₦ƛăúY˙׀·ă Ŝű ׀·f¢Σ мффлΣ !LJ2ăú2fƛŘƛă Ŝű ׀·f¢Σ ннлрΣ adz]Ŝ₦2ƛ Ŝű ׀·f¢Σ ннлсúΣ ƛyŘƛÕ׀·úƛyƎ úK׀·ű ¢wt±м ƛă ׀· úKŜ₦׀·LJŜdzűƛÕ úׁ·₦ƎŜú℗ ¢wt±м ŘŜăŜyăƛúƛÎ׀·úƛ2y˙ ØƛúK Ø׀·yƛff2ƛŘă K׀·ă LJ₦2ŘdzÔŜŘ ōŜySŦƛÕƛ׀·f ŜŦŦŜÕúă 2y˙LJ׀·úƛSyúă ØƛúK ōfl·ŘŘŜ₦ 2ØŜ₦׀·ÔúƛØƛúè ōdzű úKŜ ₦Ŝădztúă ØŜ₦Ŝ y2ű ₮dzffè ă׀·úƛă₮׀·Ôú2₦è ó/₦dzÎ Ŝű ׀·f¢Σ мффтΣ {ƛfØ׀· Ŝű ׀·f¢Σ нллúφ

/2yãƛŘŜ₦ƛyƎ úK׀·ű ƛy˙Y׀·yê õfl·ŘŘŜ₦ LJ׀·úK2f2ƎƛŜă bDC fŜØŜfă ׀·₦Ŝ ŜfŜØ׀·úŜŘ óhÕK2ŘyƛÕ]è Ŝű ׀·f¢Σ нлммΣ hÕK2ŘyƛÕ]è Ŝű ׀·f¢Σ нлмнúΣ úKŜ LJ₦ŜăŜyű ₦Ŝădztúă Y׀·è ădzƎƎŜăú úKŜ dzăŜ 2₮ ׀·ƎŜyúă Y˙2Řdzfl·úƛyƎ bDC ű2 ú₦Ŝ׀·ű õfl·ŘŘŜ₦ Řèă₮dzyÕúƛ2y℗ !ÕÕ2₦ŘƛyƎfêΣ ¢׀·yŜÎdzY׀·ōΣ ׀·y˙׀·yúƛ-bDC Y˙2y2Õf2y׀·f ׀·yűƛō2Řè úK׀·ű LJ₦ŜØŜyúă ōƛyŘƛyƎ 2₮ úKƛă b¢ ű2 ¢₦Ż!Σ K׀·ă ōŜŜy˙₦ŜÕŜyűfê ûŜăúŜŘ ƛy˙.t{κL/ LJ׀·úƛSyúă ó9Ø׀·yã Ŝű ׀·f¢Σ нлммúφ LY˙LJ₦2ØŜY˙Ŝyű 2₮ õfl·ŘŘŜ₦ LJ׀·ƛy˙׀·yŘ KèLJŜ₦׀·ÔúƛØƛúè ø׀·ă Y˙2ŘŜăú ׀·yŘ ׀·ÕÕ2YLJ׀·yÂŜŘ õè ƛY˙LJ2₦ú׀·yű ăƛŘŜ-ŜŦŦŜÕúăΣ ƛyŐfdzŘƛyƎ ØŜ₦úƛƎ2Σ LJ׀·₦׀·ŜăúKŜăƛ׀· ׀·yŘ KèLJŜ₦ŜăúKŜăƛ׀· ó9Ø׀·yã Ŝű ׀·f¢Σ нлммúΣ LJ₦ŜÕfdzŘƛyƎ úKŜ ƎŜySyŜ₦׀·fÂÎŜŘ dzăŜ 2₮ ¢׀·yŜÎdzY׀·ō ú2 ú₦Ŝ׀·ú .t{κL/φ LY˙úKƛă Ő2yúŜÈúΣ 2dz₦ ₦Ŝădztúă ăú₦Ŝăă úK׀·ű ¢wt±м ׀·yű׀·Ǝ2yƛăúă Y׀·è ōŜ ŐfƛyƛÕ׀·ffè Y˙2₦Ŝ ׀·LJLJŜ׀·fƛyƎ úK׀·y˙bDC Y˙2Řdzfl·ûƛ2y℗ LyŘŜŜŘΣ ¢wt±м ōf2Ő]׀·ŘŜ dzăƛyƎ Ő׀·LJă׀·ÎŜLJƛySƛY˙LJ₦2ØŜŘ ōfl·ŘŘŜ₦ ₮dzyÕúƛ2y˙ƛy˙₦׀·ûă øƛúK Ôèăúƛúƛă ׀·yƛY׀·fă ó5ƛyƛă Ŝű ׀·f¢Σ ннллпú℗ Ly˙׀·ŘŘƛúƛ2yΣ Dw/-снммΣ ׀·y˙2₦׀·f ¢wt±м ׀·yű׀·Ǝ2yƛăúΣ ₦ŜŘdzÔŜŘ ōfl·ŘŘŜ₦ KèLJŜ₦׀·ÕúƛØƛúè ׀·yŘ y2Èƛ2dzăƛyŁJdzű ƛyŘdzÔŜŘ ōè ÔèăúƛúƛăóK׀·₦₦dz׀· Ŝű ׀·f¢Σ ннллфú ׀·yŘ {/L ó{׀·yű2ă-{ƛfØ׀· Ŝű ׀·f¢Σ нлмнú℗ ׀Ŝy˙ŐŜΣ úKŜ ₦Ŝădztúă ŘŜăÔ₦ƛōŜŘ ƛy˙ LJdzōfƛÕ׀·úƛ2y˙ ± ădzLJLJ2₦ú úK׀·ű ¢wt±м ₦ŜÕŜLJú2₦ ƛă ׀·y˙ƛY˙LJ2₦ú׀·yű ō2ûúfŜySŐ] Õ׀·dzăŜ₦ 2₮ õfl·ŘŘŜ₦ KèLJŜ₦׀·ÕúƛØƛúè ƛy˙LJ׀·úK2f2ƎƛŜă ׀·ăă2Õƛ׀·úŜŘ øƛúK bDC 2ØŜ₦ŜÈLJ₦Ŝăăƛ2y℗ Lú ƛă ØŜ₦è fƛ]Ŝfè úK׀·ű ¢wt±м ׀·yű׀·Ǝ2yƛăúă øƛff õŜ Y˙2₦Ŝ ₮Ŝ׀·ăƛõfŜ úKŜ₦׀·LJŜdzúƛÕ 2LJúƛ2yã ƛy˙ŘŜú₦ƛY˙Ŝyű 2₮ bDC Y˙2Řdzfl·ű2₦ă℗

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!õ₦l·Yă tΣ /l·₦Ř2Î2 [Σ Cl·ff aΣ D₦ƛŦŦƛúKă 5Σ w2ăƛŜ₦ tΣ ¦fYăúŜy ¦Σ Øl·yYŜ₦₦Ŝō₦2ŜÕ] tΣ ±ƛÕú2₦ !Σ ² Ŝƛy ! óнллнú ¢KŜ ăúl·yŘl·₦Řƛăl·úƛ2y 2Ŧ úŜ₦Yƛy2f2Ǝè 2Ŧ f2øŜ₦ dz₦ƛyl·₦è ú₦l·Õú ŦdzyÕúƛ2yY ₦ŜLJ2₦ú Ŧ₦2Y úKŜ {úl·yŘl·₦Řƛăl·úƛ2y {dzō-Õ2YYƛúúŜŜ 2Ŧ úKŜ LyúŜ₦yl·úƛ2yl·f /2yúƛyŜyÕŜ {2ÕƛŜúè bŜdz₦2dz₦2f ¦₦2Řèy нмYмст-мту
!LJ2ăú2fŘƛă !Σ t2LJl·ú wΣ ¸ƛl·y∃2dz ¸Σ /2Õ]l·èyŜ 5Σ C2₦Ř ! tΣ 5l·Øƛă W.Σ 5l·ă∃dzLJúl· tΣ C2øfŜ₦ /WΣ !yl·yŘ t óнллрú 5ŜÕ₦Ŝl·ăŜŘ ăŜyã2₦è ₦ŜÕŜLJú2₦ă tн·o l·yŘ ¢wt±м ƛy ădzōdz₦2úKŜfƛl·f ySW0Ŝ ŦƛōŜ₦ă Ŧ2ff2øƛy∃ ƛyú₦l·ŘŜúNdză2₦ ƛyØŜÕúƛ2yã 2Ŧ õ2údzfƛydzY ú2Èƛy Ŧ2₦ KdzYl·y ŘŜú₦dză2₦ 2ØŜ₦l·ÕúƛØƛúè W ¦₦2f мтпYфтт-фунТ ŘƛăÕdzăăƛ2y фун-фто
!ú]ƛyã2y tWΣ /K2 /IΣ I l·yãŜy awΣ D₦ŜŜy {I óнлммú !ÕúƛØƛúè 2Ŧ l·ff WbY ƛă2Ŧ2₦Yă Õ2yú₦₦ōdzúŜă ú2 ySdz₦₦ŜƎ₦2øúK ƛy ăLJ₦₦l·f ∃l·y∃fƛ2y ySdz₦2yã l Ŝl·₦ wŜă нтуYтт-ур
.l·₦Ř2yƛ wΣ DKƛ₦₦ƛ !Σ {l·fƛ2 /Σ t₦l·yŘƛyƛ aΣ aŜ₦ƛ∃Kƛ ! óнллтú .5bC-YŜŘƛl·úŜŘ Y2ŘdzfI·úƛ2y 2Ŧ D!.! l·yŘ ∃fèÕƛyŜ ₦ŜfŜl·ăŜ ƛy Ř2₦ăl·f K2₦yfl·YƛyI· LL Ŧ₦2Y LJ2ăúyl·úl·f ₦l·úă 5ŜØ bŜdz₦2ōƛ2f стYфсл-фтр
.l·₦yl·ú aΣ 9yãfŜy lΣ t₦2LJăú CΣ 5l·Øƛă w₩Σ {2l·₦Ŝă {Σ b2úKƛl·ă C óнлмлú 5ƛăúƛyÕú ₦2fŜă 2Ŧ Õ-Wdzy b-úŜ₦YƛyI·f]ƛyl·ăŜ ƛă2Ŧ2₦Yă ƛy ySdz₦ƛúŜ ƛyƛúƛl·úƛ2y l·yŘ Ŝf2y∃l·úƛ2y ŘdzWƛy∃ l·È2yl·f ₦Ŝ∃SySŜ₦l·úƛ2y W bŜdz₦2ăÕƛ олYтулп-тумс
.ƛ₦ŘŜ₦ [!Σ bl·]l·Ydz₦l· ¸Σ Yƛăă {Σ bŜl·fŜy a[Σ .l·₦₦ƛÕ] {Σ Yl·yI·ƛ !W² l·y∃ 9Σ wdzƛÎ DΣ 5Ŝ D₦2l·ú ²/Σ !LJ2Řl·Õl· DΣ ² l·ú]ƛyã {Σ /l·úŜ₦ƛyl· aW óнллнú !fúŜ₦ŜŘ dz₦ƛyl·₦è ōfl·ŘŘŜ₦ ŦdzyÕúƛ2y ƛy YƛÔŜ fl·Õ]ƛy∃ úKŜ Øl·yƒff2ƛŘ ₦ŜÕŜLJú2₦ ¢wt±м bl·ú bŜdz₦2ăÕƛ pYурс-усл
.22₦fƛy∃ 59Σ Wl·Õ2ōăŜy l9Σ .fdzY WwΣ {KƛK !Σ .ŜÕ]Yl·y aΣ ² l·y∃ ½¸Σ ¦ŜKfƛy∃ 5¢ óнллмú LyúNl·ØŜăƛÕl·f 9ăÕKŜ₦ƛÕKƛl· Õ2fƛ fƛLJ2LJ2fèăl·ÕÕKl·₦ŘŜ ăúƛYdzfI·úŜă l·y ƛyÖ₦Ŝl·ăŜ ƛy Øfl·ŘŘŜ₦ ySW0Ŝ ∃₦2øúK Ŧl·Õú2₦ . W¦ Lyú утYсфт-тлн
.2yyƛyŭ2y WYΣ aÕbl·dz∃Kú2y t! óнллоú {ƛ∃yl·ffƛy∃ LJl·úKøl·èă ƛyØ2fØŜŘ ƛy úKŜ ăŜyãƛúăl·úƛ2y 2Ŧ Y2dzăŜ y2ÕƛÔŜLJúƛØŜ ySdz₦2yŜă ōè ySW0Ŝ ∃₦2øúK Ŧl·Õú2₦ W tKèăƛ2f ррмYпоо-ппс
/l·YŜ₦2y !!Σ {YƛúK DaΣ wl·yŘl·ff 5/Σ .₦2øy5w wl·ōÕKŜØă]è /D óнллсú DŜySúƛÔ Yl·yLJdzfI·úƛ2y 2Ŧ ƛyú₦l·ălJƛyl·f LJfl·ăúƛÕƛúè l·Ŧú Ŝ₦ ălJƛyl·f Õ2₦Ř ƛy2dz₦è l·fúŜ₦ă úKŜ ăŜØŜ₦ƛúè 2Ŧ l·dzú2y2YƛÔ ŘèăW₦ŜŦfŜÈƛl·Φ W bŜdz₦2ăÕƛ нсYнфно-нфон
/l·₦₦l·ăÕ2 a!Σ /l·ăú₦2 tΣ {ŜLJdzfØŜŘl· CW₩ ¢l·LJƛl· W/Σ Dl·úƛÕl· YΣ 5l·Øƛă aLΣ !∃dzl·è2 [D óнллтú wŜ∃dzfI·úƛ2y 2Ŧ ∃fèÕƛyŜ₦∃ƛÔ l·yŘ D!.! Ŝ₦∃ƛÔ ăèyl·LJúƛ2∃ŜySŭăƛă ōè ō₦l·ƛy-ŘŜ₦ƛØŜŘ yŜdz₦2ú₦2LJKƛÔ Ŧl·Õú2₦ ƛy ŘŜØŜf2LJƛy∃ ăLJƛyl·f ySdz₦2yãΦ bŜdz₦2ăÕƛ нсYнфно-нфонΦ
/l·₦₦l·ăÕ2 a!Σ /l·ăú₦2 tΣ {ŜLJdzfØŜŘl· C₩Σ ¢l·LJƛl· W/Σ Dl·úƛÕl· YΣ 5l·Øƛă aLΣ !∃dzl·è2 [D óнллтú wŜ∃dzfI·úƛ2y 2Ŧ ∃fèÕƛyŜ₦∃ƛÔ l·yŘ D!.! Ŝ₦∃ƛÔ ăèyl·LJúƛ2∃ŜySŭăƛă ōè ō₦l·ƛy-ŘŜ₦ƛØŜŘ yŜdz₦2ú₦2LJKƛÔ Ŧl·Õú2₦ ƛy ŘŜØŜf2LJƛy∃ ăLJƛyl·f ySdz₦2yãΦ bŜdz₦2ăÕƛ нсYнфно-нфонΦ
/l·úŜ₦ƛyl· aW₩ {ÕKdzYl·ÕKŜ₦ a!Σ ¢2YƛyI·∃l· aΣ w2ăŜy¢!Σ [ŜØƛyŜ W5Σ WdzfƛdzăΦ 5 óмффтú ¢KŜ Õl·LJăl·ƛÕƛy ₦ŜÕŜLJú2₦Y l· KŜl·ú-l·ÕúƛØl·úŜŘ ƛ2yÕKl·yySf ƛyúKŜ LJl·ƛyLJl·úKøl·èΦ bl·údz₦Ŝ оуфYумс-унпΦ
/l·dzyú /W₩ YŜèăŜ {a óнлмоú 5dzl·f-ăLJŜÕƛŦƛÕƛúè a!t]ƛyl·ăŜ LJK2ăLJKl·úl·ăŜă óaYtăúY ăKl·LJăyƎ úKŜ 2dzúÕ2YŜ 2Ŧ a!t]ƛyl·ăŜ ăƛ∃yl·ffƛy∃Φ C9.{ W нулYпуф-рлпΦ
/Kl·₦₦dzl· !Σ /₦dzÎ /5Σ /₦dzÎ C₩Σ !ØŜfƛy2 ! óнллтú ¢₦l·yãƛŜyú ₦ŜÕŜLJú2₦ LJ2úŜyúƛl·f Øl·yƛff2ƛŘ ădzōŦl·Yƛfè м ƛă ŜăăŜyúƛl·f Ŧ2₦ úKŜ ∃SyŜ₦l·úƛ2y 2Ŧ y2Èƛ2dză ōfl·ŘŘŜ₦ ƛyLJdzú l·yŘ ōfl·ŘŘŜ₦ 2ØŜ₦l·ÕúƛØƛúè ƛy ÕèăúƛúƛăΦ W ¦₦2f мттYмрот-мрпмΦ
/Kl·₦₦dzl· !Σ /₦dzÎ /5Σ bl·₦l·èl·yl·y {Σ DKl·₦l·ú [Σ Ddzffl·LJl·ffƛ {Σ /₦dzÎ C₩Σ !ØŜfƛy2 ! óнллфú Dw/-снммΣ l·ySø 2₦l·f ăLJŜÕƛŦƛÕ ¢wt±м l·yúl·∃2yăúΣ ŘŜÕ₦ŜI·ăŜă ōfl·ŘŘŜ₦ 2ØŜ₦l·ÕúƛØƛúè l·yŘ y2Èƛ2dză ōfl·ŘŘŜ₦ ƛyLJdzú ƛy Õèăúƛúƛă l·yYl·f Y2ŘŜfăΦ W ¦₦2f мумYотф-оусΦ
/K₦ƛăúYl·ă ¢W₩ w2ŘŜ W₩ /Kl·LJLJfŜ /w₩ aƒf₦2è 9W₩ ¢dz₦ySŜ₦-²l·₦ØƛÕ] w¢ óмфффлú bŜ₦ØŜ ŦƛōŜ₦ LJ₦2fƛŦŜ₦l·úƛ2y ƛy ƛyúŜ₦ăúƛúƛl·f Õèăúƛúƛă ±ƛ₦ÕK2øă !₦ÕK ! tl·úK2f !yl·ú lƛăú2LJl·úK2f пмсYппт-прмΦ
/Kdzl·y∃ l lΣ t₦ŜăÕ2úú 95Σ Y2y∃ lΣ {KƛŜfŘă {Σ W2₦Řú {9Σ .l·ăōl·dzY !LΣ /Kl·2 a±Σ Wdzfƛdză 5 óнллмú .₦l·Řè]ƛyƛy l·yŘ ySW0Ŝ ∃₦2øúK Ŧl·Õú2₦ ₦ŜfŜl·ăŜ úKŜ Õl·LJăl·ƛÕƛy₦ŜÕŜLJú2₦ Ŧ₦2Y túŘLyãópΣpútн-YŜŘƛl·úŜŘ ƛyKƛōƛúƛ2y bl·údz₦Ŝ пммYфрт-фснΦ
/2∃∃ŜăKl·ff w9 óнллрú C2ăΣ y2ÕƛÔŜLJúƛ2y l·yŘ úKŜ Ř2₦ăl·f K2₦yΦ t₦2∃ bŜdz₦2ōƛ2f ттYнфф-орнΦ
/2dzff W!Σ .Ŝ∃∃ă {Σ .2dzŘ₦Ŝl·dz 5Σ .2ƛØƛy 5Σ ¢ădzŘl· aΣ Ly2dzŜ YΣ D₦l·ØŜf /Σ {l·fúŜ₦ a²Σ 5Ŝ Y2yƛyÕ] ¸ óнллрú .5bC Ŧ₦2Y YƛÔ₦2∃fƛl· Õl·dzăŜă úKŜ ăKƛŦú ƛy ySdz₦2yl·f l·yƛ2y ∃₦l·ŘƛŜyú dzyŘŜ₦fèƛy∃ ySdz₦2LJl·úKƛÕ LJl·ƛyΦ bl·údz₦Ŝ поуYмлмт-млнмΦ

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{fl·Ŏ] {9Σ D₦ƛãű ₩Σ a l·Ŏ vΣ aŎa l·K2y˝ {.Σ tŜÎŜű { óнллрú ¢₦] . ŜÈLJ₦Ŝããƛ2y˝ l·yŘ LJK2ãLJK2-9wY l·ŎúƛØl·úƛ2y˝ õè ō₦Il·ƛy˝-ŘŜ₦ƛØŜŘ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ ƛy˝ ₦Il·ú ãLJƛy2űKl·fl·Yƛð úNl·Ŏű ySdz₦2yãΦ ₩ /2YLJ bŜdz₦2f пуфYрфр-суΦ

{fl·Ŏ] {9Σ tŜÎŜű {Σ aŎa l·K2y˝ {.Σ ¢K2YLJã2y˝ {²Σ a l·fŎl·yƎƛ2 a óнллпú .₦Il·ƛy˝-ŘŜ₦ƛØŜŘ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ ƛyŘdzŌŜã b a 5 ! ₦ŜŐŜLJű2₦ ãdzōdzyñú 2ySˆ LJK2ãLJK2₦èfl·úƛ2y˝Øƛl· 9wY l·yŘ tY/ ƛy˝űKŜ ₦Il·ű ãLJƛyl·f Ŏ2₦Ř₱ 9dz₦ ₩ bŜdz₦2ãÔƛ нлYмтсф-мттуΦ

{fl·Ŏ] {9Σ ¢K2YLJã2y˝ {² óнллнú .₦Il·ƛy˝-ŘŜ₦ƛØŜŘ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ ƛyŘdzŌŜã b a 5 ! ₦ŜŐŜLJű2₦ м LJK2ãLJK2₦èfl·úƛ2y˝ƛy˝₦Il·ú ãLJƛyl·f Ŏ2₦Ř₱ bŜdz₦2₦ŜLJ2₦ű моYмфст-мфтлΦ

{2₦ƛf [₩Σ wl·YŜ₦ [aΣ aŎtKl·ƛf [¢Σ Yl·l·y˝¢YΣ wl·YŜ₦ a{ óнллуú {LJƛyl·f ō₦Il·ƛy˝-ŘŜ₦ƛØŜŘ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ Ǝ2ØŜ₦yã ySdz₦2LJfl·ãűƛÔƛúè l·yŘ ₦ŜŐ2ØŜ₦è F₦2Y Ŏ2fŘ-Kèl JŜ₦ãŜyãƛúƛØƛúè Ŧ2ff2ø ƛyƎ Ř2₦ãl·f ₦KƛÎ2ű2Yè¢ tl·ƛy˝моyYфу-ммлΦ

{űŜƛy˝ !¢Σ ¦F₦Ŝú-±ƛyŐŜyűè / !Σ Idzl· [Σ {l·yűl·yl· [C Σ D2₦Ř2y˝ {9 óнллсú tK2ãLJK2ƛy2ãƛúƛŘŜ o-]ƛyl·ãŜ ōƛyŘã ű2 ¢wt±м l·yŘ YŜŘƛl·úŜã bDC-ãűƛY˝dzfl·ű ŜŘ ¢wt±м ú₦Il·ŦŦƛŐ]ƛyƎ ű2 úKŜ LJfl·ãY˝l· YŜY˝ō₦Il·yŜ₱ ₩ DŜy˝ tKèãƛ2f мнуYрлф-рннΦ

{Îl·ffl·ãƛ !Σ /₦dzÎ CΣ DŜLJLJŜűűƛ t óнллсú ¢wt±мY l· úKŜ₦Il·LJŜdzűƛŐ úl·₦ƎŜű Ŧ2₦ y2ØŜf l·yl·fƎŜãƛŐ Ř₦dzƎãK ¢₦ŜyŘã a2f aŜŘ мнYрпр-ррпΦ

¢2YƛYl·Ǝl· aΣ /l·űŜ₦ƛYl· a₩ óнллпú ¢KŜ₦Y˝2ãŜyãl·úƛ2y˝l·yŘ LJl·ƛy₱ ₩ bŜdz₦2ōƛ2f смYо-мн Φ

¢2YƛYl·Ǝl· aΣ /l·űŜ₦ƛYl· a₩Σ al·fYōŜ₦Ǝ !.Σ w2ãŜy˝ ¢!Σ DƛfōŜ₦ű lΣ {]ƛyŷŜ₦ YΣ wl·dzYl·yŷ˝ .9Σ .l·ãōl·dzY˝ !LΣ ₩dzfŧdzã 5 óмффуú ¢KŜ Ŏf2ySŘ Ŏl·LJãl·ƛŎƛy˝ ₦ŜŐŜLJű2₦ ƛyűŜƎ₦l·úŜã Y˝dzfűƛLJfŜ LJl·ƛy˝-LJ₦2ŘdzŎƛyƎ ãűƛY˝dzfŧ₱ bŜdz₦2y˝нмYром-рпоΦ

±l·Ø₦Ŝ] w Σ tŜl·₦ãŜ 55Σ C2dzl·Ř Y óнллтú bŜdz₦2yl·f LJ2LJdzfl·úƛ2yã Ŏl·LJl·ōfŜ 2Ŧ ₦ŜƎŜySŜ₦l·úƛ2y˝Ŧ2ff2øƛyƎ l·Ŏ2YōƛySŘ ú₦ŜIl·ű˝Syű ƛy˝₦Il·úã øƛúK ãLJƛyl·f Ŏ2₦Ř ú₦Il·yãŜŎűƛ2y₱ ₩ bŜdz₦2ú₦Il·dzY l· нпYмсст-мстоΦ

±ƛÎÎl·₦Ř a ! óнлллú /Kl·yƎŜã ƛy˝ dz₦ƛyl·₦è ōfl·ŘŘŜ₦ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ Ywb ! l·yŘ bDC LJ₦2ű Ŝƛy˝ Ŧ2ff2øƛyƎ dz₦ƛyl·₦è ōfl·ŘŘŜ₦ Řèãŧdzyðúƛ2y₱ 9ÈLJ bŜdz₦2f мсмYнто-нуп Φ

±ƛÎÎl·₦Ř a ! óнллсú bŜdz₦2ŐKŜY˝ƛŎl·f LJfl·ãűƛÕƛúè l·yŘ úKŜ ₦2fŜ 2Ŧ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ã ƛy˝ ōfl·ŘŘŜ₦ ₦ŜŦfŜÈ LJl·úKøl·èã l·ŦűŜ₦ ãLJƛyl·f Ŏ2₦Ř ƛy2dz₦è₱ t₦2Ǝ .₦Il·ƛy˝wŜã мрнYфт-ммр Φ

² Ŝl·ØŜ₦ [/Σ ±Ŝ₦ƎKŜãŜ tΣ .₦dzŐŜ ₩/Σ CŜKfƛyƎã aDΣ Y₦ŜyÎ bwΣ al·₦ãK 5w óнллмú !dzű2y2YƛÔ Řèã₦ŜŦfŜÈƛl· l·yŘ LJ₦ƛYl·₦è l·ŦŦŜ₦Syű ãLJ₦2dzűƛyƎ l·ŦűŜ₦ ŐfƛLJ-Ŏ2YLJ₦Ŝãã2y˝ ƛy2dz₦è 2Ŧ úKŜ ₦Il·ű ãLJƛyl·f Ŏ2₦Ř₱ ₩ bŜdz₦2ú₦Il·dzY l· муYммлт-ммм ф Φ

·dzŜ vΣ ₩2yƎ .Σ /KŜy˝¢Σ {ŐKdzYl·ŐKŜ₦ a ! óнллтú ¢₦l·yãÕ₦ƛLJűƛ2y˝ 2Ŧ ₦Il·ű ¢wt±м dzűƛfƛÎŜã l· Řdzl·f LJ₦2Y2űŜ₦ ãèãűŜY˝ úKl·ű ƛã LJ2ãƛúƛØŜfè ₦ŜƎdzfl·úŜŘ ōè ySNØŜ Ǝ₦2øúK Ŧl·Ŏű2₦Φ ₩ bŜdz₦2ŐKŜY˝ млмYнмн-ннн Φ

½K2dz ·CΣ wdzãK w! ómффсú 9yŘ2ƎŜy2dzã ō₦Il·ƛy˝-ŘŜ₦ƛØŜŘ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ ƛã l·yűŜ₦2Ǝ₦l·ŘŜfè ú₦Il·yãLJ2₦ű˝ŜŘ ƛy˝LJ₦ƛY˝l·₦è ãŜya2₦è ySdz₦2yãΦ bŜdz₦2ãÕƛŜyŐŜ тпYфпр-фро Φ

½Kdz ²Σ hÈŦ2₦Ř D{ óнллтú tK2ãLJK2ƛy2ãƛúƛŘŜ-o-]ƛyl·ãŜ l·yŘ Yƛű2ƎŜy˝ l·ŐűƛØl·úŜŘ LJ₦2ű Ŝƛy˝]ƛyl·ãŜ ãƛƎyl·fƛyƎ LJl·úKøl·èã YŜŘƛl·úŜ l·Ŏdzű Ŝ bDC ãŜyãƛúƛÎl·úƛ2y˝ 2Ŧ ¢wt±м Φ a2f /Ŝff bŜdz₦2ãÕƛ опYсуф-тллΦ

½Kdz ½²Σ C₦ƛŜãã lΣ ²l·yƎ [Σ ½ƛYY˝Ŝ₦Y˝l·yŷ˝ !Σ .dzŐKfŜ₦ a ² óнллмú .₦Il·ƛy˝-ŘŜ₦ƛØŜŘ ySdz₦2ú₦2LJKƛÔ Ŧl·Ŏű2₦ ó.5bCú ƛã dzLJ₦ŜƎdzfl·úŜŘ l·yŘ l·ãä2Ŏƛl·úŜŘ øƛúK LJl·ƛy˝ƛy˝ŐK₦2yñÕ LJl·yŐ₦Ŝl·úƛúƛã Φ 5ƛƎ 5ƛã {Ŏƛ псYмсоо-мсоф Φ